Official Organ of the Society for the Study of Development and Growth

GROWTH

A JOURNAL FOR STUDIES OF DEVELOPMENT AND INCREASE

VOLUME V

PAPERS 153 - 189

1941

(Printed in U.S.A.)

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The Heffernan Press, 150 Fremont St., Worcester, Mass.

Direct
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GROWTH

A JOURNAL FOR STUDIES OF DEVELOPMENT AND INCREASE

VOLUME V, NUMBER 1
PAPERS 153-161

MARCH, 1941 $5.00 A YEAR

GROWTH

A journal for the coördination of studies of increase and development as general properties of nature. Published by contributors and subscribers on a coöperative, non-profit, non-salary basis. The field of investigation is primary and work will be published in which the approach is through mathematics, physics, chemistry, genetics, embryology, philosophy, and anthropology; just as will studies of development in its several expressions, and of mass increase and the part played therein by nutrition, vitamines, and hormones.

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Growth, 1941, **5**, 1-9.

RELATIVE GROWTH IN BANTAMS AND LEGHORNS*[1]

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It has been previously reported by the writer (Lerner, 1937) that the ontogenetic relative growth constants for the weight of the breast muscle and the length of the long bones of the hind limb are the same in different breeds of poultry. This conclusion was based on dissections of 760 Barred Plymouth Rocks, Black Minorcas, first crosses between them and backcrosses. A further comparison of the length of the tarsometatarsus of representatives of several other breeds and of jungle fowl in relation to body weight, suggested that a uniform morphogenetic pattern controlling size relationships of parts exists within the species *Gallus gallus*. Such a situation has been reported by Hersh (1934) with respect to skull proportions within genera of extinct titanotheres and by others since, with respect to different structures in various species. There was, however, an exception to the suggested plan for chickens, since limited data on Bantams failed to conform to the growth patterns for the larger-sized breeds.

This was considered by the writer to be a type of intraspecific variation of the kind later demonstrated to exist in dogs by Lumer (1940). All of the breeds of dogs investigated by him failed to conform to a single pattern with respect to their skull measurements and it was possible to classify some 28 breeds into six distinct allometric tribes, characterized by differences in their relative growth constants. Lumer's analysis was based on the measurements made on dog skeletons and presumably full-grown dogs were largely used. Hence the relative growth constants investigated by him referred to factors controlling final proportions rather than to those controlling relations between the ontogenetic rates of growth of parts. The distinction between these two types has been appreciated by Huxley (1932), although the fact that the ontogenetic constants are not necessarily the same as those governing final proportions has not been particularly stressed. Recently, Needham and Lerner (1940) proposed a revision of the terminology of relative growth, suggesting the

*Received for publication on November 4, 1940.
[1]Assistance in computations has been provided under the auspices of *WPA* Official Project No. 65-1-08-91, Unit B6.

term *heterauxesis* to describe the actual relative growth of parts in relation to the whole or other parts, and the term *allometry* for relative growth relationships between groups or individuals compared at maturity or at any other given stage of development. According to this terminology in the first type of comparison the values above unity indicate positive relative growth or *tachyauxesis;* those equal to unity,—*isauxesis;* those below unity,—*bradyauxesis.*

In the writer's analysis of the relative growth of chickens, practically all the Bantams used were mature, while the majority of the Barred Plymouth Rocks and the Black Minorcas were in the process of rapid size increases. Therefore, if the allometric constants in this case differ from the constants of heterauxesis and are lower, a difference in growth pattern between the Bantams and the larger-sized breeds has not been necessarily demonstrated. The present report is devoted to an examination of this question on the basis of data obtained from dissection of 179 Single Comb White Leghorns, Rose Comb Black Bantams, and reciprocal crosses between them.

Materials and Methods

The Leghorns used in this study originated from the University of California flock. Eggs from a Rose Comb Black Bantam flock purchased from a private breeder several years ago produced the foundation stock for the group of Bantams studied. Although the birds hatched from these eggs were of the type prescribed by the Standard of Perfection of the American Poultry Association (1930), in future generations single comb segregates appeared. Nevertheless from the standpoint of size and conformation the stock was representative of the Bantam class. The standard weight for mature Bantam males is 26 ounces, for females 22 ounces, whereas the mature Leghorns should, according to the standard, weigh 6 and 4½ pounds respectively.

The purebred stock was produced from natural matings; for the production of crosses artificial insemination was practiced to insure fertility. Four savings of eggs were set at weekly intervals and hatched in March and April. At the time of hatching all of the chicks were wing-banded. At predetermined ages one-fourth of the chicks from each type of mating in Hatches 1 and 3, and one-third of the chicks from each type of mating in Hatches 2 and 4 were killed and dissected. Thus, Hatches 1 and 3 provided chicks for four dissections each (at 4, 8, 16, and 24 weeks of age), and Hatches 2 and 4 for three dissections each (at 2, 6, and 20 weeks of age).

The wing-band numbers were assigned for each dissection group at the time of hatching. This schedule was adhered to except in cases where the chicks died before the age at which they were slated for dissection. Out of the 196 chicks hatched for this experiment, 179 were eventually used in the analysis. The difference of 17 is accounted for by mortality and by the few chicks whose bones were accidentally destroyed or damaged in preparation for measurement.

The methods of dissection and measurement, as well as of calculation of the relative growth constants have been described in an earlier publication in detail (Lerner, 1937).

Heterauxesis

Table 1 presents the values of a and b in the relative growth equation

$$y = bx^a,$$

where x stands for body weight, y for the respective part under consideration, b for the initial growth index, and a for the actual equilibrium constant or growth ratio. The a value in the case of allometry is to be referred to as the limiting equilibrium constant or ratio. These terms are used as suggested by Huxley and Teissier (1936). The question whether a for individuals is really constant or not has been previously discussed (Lerner, 1938). While in individual birds the value of a changes during the growth process, the growth ratios presented here are to be considered as average values, representing the average ratio of growth rates of the parts to that of the whole for the period studied (2 to 24 weeks of age). It should also be noted that the dimensions on the two sides of the equation relating the length of leg bones to body weight are not equal. Isauxesis, or equality of dimensionally corrected growth rates of the whole and the part, in this relation is represented by an a value of 0.333 and not of unity as in case of the pectoralis major. A number of conclusions emerge from Table 1:

1. The growth ratios (a) of the pectoralis major (one side only weighed), the femur, the tibiotarsus, and the tarsometatarsus with respect to body weight of the Bantams do not differ from those of the Leghorns. Their values are remarkably close to those previously reported for Barred Plymouth Rocks, Minorcas, and their hybrids.

2. The initial growth index (b) for each structure varies inversely with the magnitude of a. This phenomenon has been analyzed in detail by Lumer (1939). For the present purpose it is sufficient to point out that this reciprocal relation may account for the higher

TABLE 1

CONSTANTS OF HETERAUXESIS IN LEGHORNS, BANTAMS, AND CROSSES

Sex	Mating	Number	Pectoralis major		Femur		Tibiotarsus		Tarsometatarsus	
			a	*b*	*a*	*b*	*a*	*b*	*a*	*b*
Male	S. C. W. Leghorn	58	1.224 ± .12	.009	.374 ± .03	5.62	.413 ± .04	6.15	.422 ± .05	4.17
	R. C. B. Bantam	17	1.407 ± .09	.005	.355 ± .05	6.03	.383 ± .03	7.17	.383 ± .05	4.89
	Bantam♀ × Leghorn♂	4	1.242 ± .04	.009	.362 ± .03	5.91	.391 ± .05	6.92	.374 ± .06	5.31
	Leghorn♀ × Bantam♂	25	1.224 ± .12	.011	.392 ± .04	4.88	.426 ± .05	5.63	.430 ± .05	3.84
	All males	104	1.213 ± .14	.010	.377 ± .04	5.45	.409 ± .04	6.28	.422 ± .05	4.13
Female	S. C. W. Leghorn	43	1.273 ± .16	.007	.383 ± .04	5.34	.413 ± .04	6.22	.407 ± .05	4.48
	R. C. B. Bantam	10	1.380 ± .09	.006	.408 ± .15	4.55	.382 ± .10	7.25	.410 ± .06	4.02
	Bantam♀ × Leghorn♂	5	1.191 ± .05	.015	.358 ± .01	6.29	.410 ± .03	6.48	.398 ± .03	4.79
	Leghorn♀ × Bantam♂	17	1.237 ± .17	.011	.372 ± .04	5.40	.405 ± .05	6.12	.397 ± .04	4.47
	All females	75	1.240 ± .21	.010	.386 ± .07	5.15	.407 ± .05	6.36	.415 ± .05	4.19

(though insignificantly so) magnitudes of a for the pectoralis major of the Bantams as compared to the Leghorns, if b and not a is the fixed parameter of the relative growth equation. This is not an unreasonable assumption in view of Pontecorvo's (1938) suggestion that the initial growth index is the hereditary factor determining conformation.

3. While a distal-proximal gradient in the leg may be noted when all males or all females are considered (vide Lerner, 1937), this gradient is not expressed in all of the sub-groups. This is in accordance with the observations of Clark and Hersh (1939) on growth gradients in the backswimmer.

4. The magnitude of the initial growth index in the leg bones follows, as expected, the original order in length of bones. Towards the end of the growth period the tarsometatarsus, due to its higher growth rate, exceeds the femur in length. At two weeks of age, however, the femur is still the longer of these two bones.

It then appears that tachyauxesis is demonstrated for the structures studied in the Bantam as well as in breeds of larger size. The uniformity of the morphogenetic plan of growth in the different breeds of chickens is thus confirmed with the amendment, however, that the Bantams, contrary to the original suggestion, also adhere to this plan.

Allometry

In order to explain the basis for the erroneous assignment of the Bantams to a different tribe (in Lumer's sense) from the "normal" sized birds an allometric analysis in addition to the above discussion on heterauxesis is necessary. Jaap and Penquite (1938) have pointed out that in the chicken skeletal growth ceases earlier than does the growth of the body as a whole. Limited observations on those birds in this study which were carried past 20 weeks of age tend to support this statement. Consecutive measurements of shank length at weekly intervals failed to show any increases, while increments in body weight were still being observed.

It has been shown by Lumer (1937) that the relative growth equation is not valid for the entire duration of growth. Deviations from a straight line on a log-log plot of y against x are to be expected when these variables begin to approach their final values. One possibility is that the time of cessation of skeletal growth in the Bantams is earlier than in the Leghorns. Another alternative, however, is that the actual growth ratios are higher than the limiting growth ratios

in both breeds. This would follow under the conditions of tachyauxesis, as shown by Lumer's (1937) mathematical analysis. Then the original interpretation arose as a result of comparison of the limiting growth ratio of the Bantams with the actual growth ratio of the other breeds. Verification of this suggestion is possible by comparing the observed proportions of representatives of the two breeds at different ages with the proportions calculated on the basis of a and b in Table 1. Regression equations were accordingly used to obtain the calculated values (Table 2).

TABLE 2

y	Males	Females
Pectoralis major	$\log y = 1.2132 \log x - 0.9930$	$\log y = 1.2395 \log x - 1.1051$
Femur	$\log y = 0.3768 \log x + 0.7363$	$\log y = 0.3864 \log x + 0.7120$
Tibiotarsus	$\log y = 0.4094 \log x + 0.7977$	$\log y = 0.4067 \log x + 0.8034$
Tarsometatarsus	$\log y = 0.4216 \log x + 0.6160$	$\log y = 0.4148 \log x + 0.6217$

The differences between the values thus obtained and the actual values of the respective y's for the three last age groups are presented in Table 3. The offspring of the mating between Bantam females and Leghorn males are omitted, because, due to the small number of birds in this cross, representation in the age groups considered is not complete.

The situation revealed in Table 3 is quite different for the breast muscle than for the leg bones. Comparing the extreme case for the latter, that of the tarsometatarsus, with the pectoralis major, it is seen that whereas observed values of the muscle exceed the calculated values in the Bantams, the contrary is true for the bone. In the case of the Leghorns the situation is completely reversed up to 20 weeks of age, while the crosses occupy an intermediate position in this respect.

The interpretation here is quite obvious. So far as the tarsometatarsus is concerned the cessation or slowing down of bone growth in the Bantams is responsible for the fact that the limiting equilibrium constant is reached at a body weight, at which growth of the Leghorns is still expressed by the actual equilibrium constant. Furthermore since the observed values in the Leghorns do not become lower than the calculated values until 24 weeks of age, the limiting ratio is reached in them later than in the Bantams. Thus the explanation for the Bantam—"normal-sized" breeds relationship suggested above is verified.

With regard to the pectoralis major the Leghorn-Bantam relation-

TABLE 3

EXCESS OF THE OBSERVED OVER THE CALCULATED VALUES

(Pectoralis major in gms.; bones in mm.)

	Age in weeks														
	Number			Pectoralis major			Femur			Tibiotarsus			Tarsometatarsus		
	16	20	24	16	20	24	16	20	24	16	20	24	16	20	24
Leghorn males	9	9	7	—5.5	—3.8	—14.1	1.1	1.3	—0.9	3.1	2.3	—0.1	2.6	1.7	1.6
Leghorn females	5	4	7	—0.1	—2.9	—17.3	2.2	4.0	—2.3	6.7	4.5	—1.0	4.5	2.8	—2.0
Bantam males	2	1	2	5.8	9.0	3.9	—2.2	—3.7	—2.0	—0.8	—4.9	—1.9	—2.9	—5.7	—2.6
Bantam females	2	2	1	7.3	3.8	4.2	1.6	0.2	—4.0	—0.1	1.5	—4.1	—2.3	—3.2	—5.0
(L♀ × B♂) males	3	4	2	0.3	5.7	—2.2	0.6	0.8	—5.2	4.6	4.9	—2.6	1.6	3.3	—7.4
(L♀ × B♂) females	1	1	1	6.8	6.1	—2.5	0.8	—2.9	—6.0	—0.6	—4.1	—6.8	—0.7	—2.9	—4.8

ship is of a different order. Here it is the Bantams which show an excess of the observed over the calculated values. This can only mean that while in the Leghorns the increase in the weight of the breast muscle with respect to the increase in body weight has slowed down as compared to the rate of the process in earlier stages of growth, in the Bantams the pectoralis major is still exhibiting high tachyauxesis.

The above strongly indicates that the time relationships involved need be considered in the elaboration of problems of relative growth. The Bantams at their mature weight do not exhibit the same phenomena as the Leghorns at a comparable weight. A slowing down of the relative rate of bone growth as maturity is being reached is found in the Bantams. At the same weight the Leghorns are still in a very active phase of bone growth. On the other hand, the accretion in muscle weight proceeds at a different relative rate in the Bantams than it does in the Leghorns as both approach maturity. Hence both age and size are involved at every given point of comparison. Age was not at all taken in account in the previously presented plot of tarsometatarsal length against body weight of different breeds and species of fowl (Lerner, 1937, Figure 3, p. 541). Besides the Bantams this figure contained five points for growing birds and ten points for fully grown ones. Hence it is to be considered as a compound plot. The intriguing question why the jungle fowl exceeded the Bantams in tarsometatarsal length at the same body weight and presumably at the same stage of maturity is still open. From the material presented here, however, it is clear that while the constants governing final proportions may differ in the Bantams from those in larger-sized breeds, the average constants of ontogenetic relative growth of breast muscle and leg bones are the same in all of the breeds of chickens investigated.

Summary

A study of the relative growth in Single Comb White Leghorns, Rose Comb Black Bantams, and crosses between them, reveals that the constants of heterauxesis for the breast muscle and leg bones in the Bantams are of the same magnitude as in the Leghorns and the other breeds studied previously. The limiting equilibrium constants for bone length are lower in the Bantams than the actual equilibrium constants. Hence separate comparisons are necessary for allometry and for heterauxesis. From the latter standpoint the uniform morphogenetic plan of growth postulated for the larger-sized breeds of chickens applies to Bantams as well. Certain considerations regarding

age and size relationships in studies of relative growth are also presented.

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Growth, 1941, **5**, 11-18.

THE INFLUENCE OF l-PROLINE ON LARVAL GROWTH IN DROSOPHILA MELANOGASTER*

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During the search for rôles played in growth by naturally occurring tissue components, Hammett and Collings (1937) found that l-proline is effective in enhancing differentiation in *Obelia geniculata*. That l-proline forwards differentiation expression has also been demonstrated in *Pagurus* (Hammett, Hammett, and Goldsmith, 1940), and in rabbit ova *in vitro* (Miller and Reimann, 1941). The present experiments were, therefore planned to test whether l-proline would have a similar action in *Drosophila melanogaster* larvae.

Growth in most insects is decidedly cyclical in that periods of active increase in mass are interrupted by ecdyses, a fact well illustrated by the apodous oligopod larva of the Dipteran, *Drosophila*. These fast growing larvae are most efficient nutriment absorbing machines, capable of synthesizing within four or five days sufficient material for initiation and continuance of maturation within the puparium. Two moults which involve slight but definite structural changes occur before pupation. In the *Calliphorid Diptera* the prematuration period is for the most part spent within the egg so that few structures are formed *de nova* during the larval stages. On the other hand, a certain amount of differentiation does occur. For present purposes such differentiation may be defined as cellular and structural specialization.

The following facts indicate the manner in which differentiation occurs in *Drosophila* larvae. Evidence for the existence of a pupation hormone has been obtained for *Drosophila* by Bodenstein (1938). This hormone is known to be elaborated by the ring gland, but whether the same or a different structure is involved in hormonal control of larval moults is not known. That a moulting hormone is concerned in the initiation of ecdysis of hemimetabolous insects has been rather generally accepted (Craig and Hoskins, 1940). The

*Received for publication on December 30, 1940.

[1]I wish to thank Dr. Frederick S. Hammett and the Staff of the Lankenau Hospital for criticism and aid in making this work possible, and Miss Lorraine Schader of Wellesley College for technical assistance.

moulting process, then, may involve differentiation in that certain cells must develop the ability to elaborate the hormone. In addition during moulting there is a change in form, which in this case is small, but which is accompanied by reconstruction of the epidermal cells. Also in the *Diptera,* imaginal buds differentiate throughout the larval stages. In view of these facts it seems logical to assume that any growth factor which hastens moulting must necessarily stimulate differentiation. The indices of differentiation growth here measured are body length and duration of the larval periods.

Materials and Methods

Swedish-b wild type of *Drosophila melanogaster,* reared on banana agar medium at 25°C. was used in these experiments. Eggs were collected and sterilized as previously described (Wilson, 1939). More recent experimentation shows that viable eggs may be obtained even after 30 to 50 minutes immersion in 85 per cent alcohol. This latter sterilization procedure was followed in the present experiments.

Larvae were reared in an agar, salt, sugar medium modified from that of Thorpe (1939) by Miss Lorraine Schader of this laboratory (1941). The medium is acid enough for normal larval development but still may be autoclaved. No precipitation of salt occurs. To this mixture was added three grams of Fleischmann's fresh baker's yeast per 100 cc. of culture. This amount of yeast is about half enough to produce pupation within 128 hours after egg laying. Under these conditions the first stadium was prolonged four hours and the second about five more hours. The smaller amount of yeast was thought advisable in order to be sure that the l-proline would not already be in excess.

The culture medium including the yeast was autoclaved 20 minutes at 115 pounds pressure 121°C. and pipetted into 5 cm. petri dishes which served as culture dishes. Ten experimental and 10 control dishes were prepared for each experiment. Approximately 20 eggs were added to each dish directly from 80 per cent alcohol by means of a bulb pipette. The subsequent larval number varied due to irregular egg hatching.

Before pouring, aliquot portions of l-proline solution were thoroughly mixed with one-half of the culture medium for the test. Fresh stock solutions of l-proline were made each day from a Hoffman La Roche product purified by Mr. Thomas Callan of the Lankenau Hospital Research Institute. To prepare the stock solution 0.057 gram of l-proline was dissolved in 2 cc. of 85 per cent alcohol (to

sterilize) and added to 100 cc. of autoclaved distilled water. Equal amounts of alcohol were added to control media.

Tests and controls were run simultaneously in an incubator at 25±0.2°C. Culture dishes for each experiment were placed in sterilized evaporating dishes and not opened until the conclusion of the test. Larvae were killed during two periods, five sets of dishes each time. The first period was at 52 hours from the median hour of egg laying at which time part of the animals in the control dishes had moulted into the second stadium. The second period was at the time of partial ecdysis of control animals into the third stadium at about 80 hours after egg laying.

Normal growing larvae begin to form second instars at 44 hours, and more than 99 per cent are in the second instar at 60 hours; while the second instar animals start to moult at 68 hours and are almost all in the third instar at 88 hours. With the lower percentage of yeast used in this experiment the same type of distribution occurs but about five hours later. The difference between test and control may be determined at any time during this period, but was invariably taken before 60 hours for the first period and before 90 hours after egg laying for the second period to insure significant comparison.

For observation, the larvae were killed as described by Alpatov (1929). The length of the mouth parts and appearance of the anterior spiracles as developed by him are used as indices. Measurements were made with a micrometer in a 10X ocular used with a 48mm. objective.

Every effort was made to insure absolute sterility. Meticulous care was used to prevent the appearance of yeast and mould in the laboratory or incubator. All the glassware was autoclaved and in addition placed in the dry oven at 150°C. for at least an hour. Turbidity tests with bacto-peptone glucose broth were made on each culture and contaminated dishes discarded. These precautions are essential, for growing yeast provides growth factors incompatible with the conditions of this experiment.

Discussion and Results

Table 1 shows the influence of l-proline on time of moulting from first to second instars in *Drosophila* larvae, while Table 2 shows the influence of l-proline in subnutritional doses on moulting from second to third instars. As has been reported for l-proline as well as other amino acids, there is here an ineffective concentration, a range of ecdysis-stimulating concentrations and a range of moulting inhibitory

TABLE 1

THE INFLUENCE OF l-PROLINE ON MOULTING FROM FIRST TO SECOND INSTARS IN DROSOPHILA LARVAE

l-proline concentr. in M/	No. of Expts.	No. of individuals used		No. of expts. with more 2nd instars		Total % difference between number of 2nd instars in test compared to control
		Control	Test	Test	Control	
5,000	2	96	90	0	2	—32.5
10,000	8	520	555	2	6	— 4.9
25,000	7	770	773	3	4	— 4.9
50,000	5	621	576	5	0	+13.0
100,000	5	707	615	3	2	+ 6.3
200,000	4	434	385	2	2	— 2.5

TABLE 2

THE INFLUENCE OF l-PROLINE ON MOULTING FROM SECOND TO THIRD INSTARS IN DROSOPHILA LARVAE

l-proline concentr. in M/	No. of Expts.	No. of individuals used		No. of expts. with more 3rd instars		Total % difference between number of 3rd instars in test compared to control
		Control	Test	Test	Control	
5,000	2	102	104	0	2	
10.000	5	512	555	1	4	— 7.8
25,000	5	455	461	0	5	—11.6
50,000	5	422	561	5	0	+13.6
100,000	5	501	486	3	2	+ 1.9
200.000	4	461	496	2	2	+ 1.6

concentrations. This latter may be considered a toxic range in so far as growth is concerned; but of course, not necessarily toxic to the point of lethal action. These effects are illustrated by the total per cent of difference indicated in Figure 1 by circles, and on the tables in the last columns. M/5,000 concentration is toxic since animals so treated failed to reach the third instar in any reasonable time. M/10,000 is also significantly toxic to moulting while M/25,000 is more retardant at 80 hours than at 52. M/50,000 is stimulating, since animals so treated invariably showed shorter moulting periods in the tests when compared to controls. A concentration of M/100,000 may be considered slightly stimulating and M/200,000 not significantly so.

The only other efforts to analyze amino acid requirements of *Drosophila* have been those of van't Hoog (1935), Lafon (1939), and Tatum (1939). Van't Hoog demonstrated the need of cystine and tryptophane for pupation within the normal period. Tatum found it necessary to add tryptophane to hydrolyzed casein for normal growth. Lafon's work is more extensive, but since he does not add

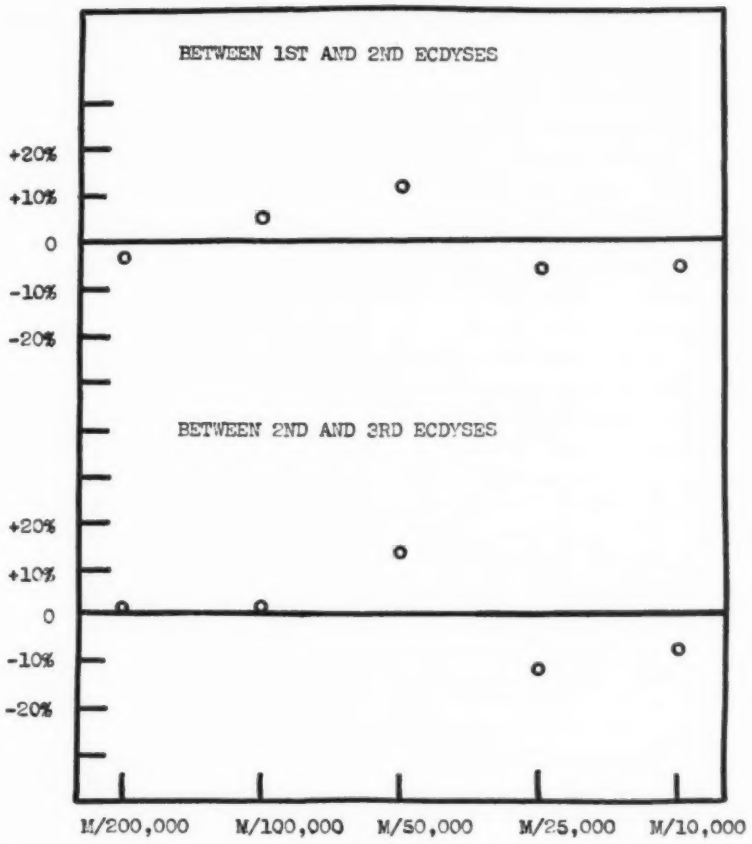

FIGURE 1

THE PERCENTAGE DIFFERENCE BETWEEN TEST AND CONTROL ECDYSES FOR THE STATED CONCENTRATIONS

required vitamins of the *B*-complex and depends entirely on Nipagine for control of microorganisms, his work is open to doubt. He finds that tryptophane and cystine are essential for larval growth, and arginine and histidine for puparium formation. He was unable to obtain normal growth with a mixture of 18 amino acids including threonine unless beef peptone was added. None of these investigators mentioned l-proline specifically, nor do they study larval growth critically.

Results with one amino acid are not conclusive unless corroborated by work with other forms and compared with other amino acids. The addition of l-proline in concentrations of M/50,000 to M/100,000 stimulates developmental growth process in *Drosophila* larvae, and appears to stimulate the specific process of differentiation as indicated by shorter stadia. A like result was obtained with oxyproline (Hammett, 1937), glutamic acid (Hammett, 1936), and aspartic acid (Hammett and Schlumberger, 1937). All these compounds are related through the production of pyrrolidone in intermediary metabolism. From this Hammett has postulated that it is not l-proline as such which is the effective group in differentiation, but pyrrolidone or some derivative thereof. The validity of the assumption is supported by the results of Miller and Reimann (1941) with rabbit ova *in vitro*, and the results of Hammett, Hammett, and Goldsmith (1940) with the regenerating chela of *Pagurus*. In both cases differentiation expression was specifically forwarded, and differentiation expression alone. My results, showing as they do a shortening of the periods for ecdysis, appear to demonstrate that here too in *Drosophila* larval growth the amino acid l-proline produces stimulation of this specific process of differentiation. If this is so, l-proline would probably exert its effect either through specific hormone elaborating cells or as a factor in general differentiation of epidermal tissue.

In order to answer the question as to whether animals moulting earlier show size difference from normal, measurements of length were made. Table 3 shows the results of such measurements on animals

TABLE 3

AVERAGE LENGTH IN MM. OF DROSOPHILA LARVAE TREATED WITH M/50,000 l-PROLINE

Exp.	52 hours		80 hours approx.	
	1st instar	2nd instar	2nd instar	3rd instar
1 Control	1.58	1.95	3.03	4.05
Test	1.62	1.88	3.05	3.72
2 Control	1.57	1.85	2.98	3.50
Test	1.55	1.93	3.02	3.63
3 Control	1.58	2.07	2.97	3.78
Test	1.52	1.98	3.01	3.73
4 Control	1.53	1.93	3.10	3.71
Test	1.58	1.91	3.05	3.53
5 Control	1.63	1.97	2.87	3.69
Test	1.64	1.93	3.11	3.86

in which the greatest stimulation to ecdysis was obtained: namely, those treated with M/50,000 l-proline. No significant difference is shown in length between test and control, nor is there any significant trend difference.

It has been shown by Alpatov (1929) that *Drosophila* larvae decrease rate of length growth as ecdysis approaches. In fact larvae which remain in the lower stadium throughout the period of about 15 hours during which moulting is occurring are nearly all the same size. These experiments seem to indicate that l-proline has no effect on length growth but merely shortens the latent period before ecdysis, after a definite length has been reached. Observation of the animals which had moulted earlier due to the presence of l-proline gave the impression that the chitin was more fragile. Distortion was more common. Perhaps moulting had occurred before sufficiently resistant chitin had been laid down.

Summary

The effect of l-proline in concentrations ranging from M/200,000 to M/5,000 on differentiation of *Drosophila melanogaster* larvae, as measured by duration of stadia, was investigated. It was found that the duration of first and second instars was reduced by exposure to concentration of M/50,000 to M/100,000. The duration of these same stages was prolonged by exposure to M/25,000 or higher concentrations of l-proline. Also, l-proline does not affect the length growth but rather shortens the latent period before ecdysis after maximum growth of each instar has been attained.

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Growth, 1941, **5**, 19-26.

THE EFFECT OF VITAMIN B_1 (THIAMIN) ON THE BODY LENGTH AND DURATION OF THE LARVAL PERIOD IN DROSOPHILA MELANOGASTER*

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INTRODUCTION

The effect of thiamin on the growth of insects has been studied by many investigators, at least three of whom have observed it in connection with the growth of Drosophila larvae. The reports of these three authors are not in agreement, however. Tatum (1939) and van't Hoog (1935) consider thiamin essential, while Lafon (1939) maintains that it is not necessary for the full development of Drosophila. In this study, an attempt was made to determine the effect, if any, of this vitamin on the growth of a wild type, Swedish b, *Drosophila melanogaster*. In this case, however, an index of determination, different from that of previous investigators, was used. Instead of merely observing the time of development from egg to pupa to imago, these observations were supplemented with actual measurements of body length of the three stages or instars of larval development. In this way it was possible to determine where in the life cycle thiamin took its effect and also to what extent this effect was evident.

METHODS

For this investigation, a culture medium for Drosophila larvae must fulfill three important requirements. First, the medium must support the growth of the larvae, but should not allow the development of microorganisms, which might serve as food for the larvae. Second, thiamin must be removed from the medium, but all other constituents should be left intact. Third, the thiamin must be replaced, so that its effect can be observed. Therefore, a medium suggested by van't Hoog (1935) was adopted. This medium contained agar, sucrose, yeast, and Pearl's salt mixture S-101, made up in distilled water. Thorpe (1939) also used this medium, but with the salt mixture modified by J. H. Sang. For this study, it was found more convenient to use the salt mixture as modified by Sang. However, after pre-

*Received for publication on January 20, 1941.

paring the solution, it was discovered that, upon standing, certain salts precipitated out, forming large crystals. Also, the solution was so strongly acid that when it was autoclaved with the other constituents of the medium, the agar was hydrolyzed and did not harden. Therefore, it was necessary to divide the salt solution into three parts. The final method of preparation is given in Table 1.

TABLE 1

Solution A	*Solution B*
8 g. potassium sodium tartrate	5 g. tartaric acid
2 g. ammonium sulfate	0.65 g. potassium dihydrogen phosphate
0.5 g. magnesium sulfate	250 cc. distilled water
250 cc. distilled water	
Solution C	
0.25 g. calcium chloride	
250 cc. distilled water	

Solutions *A*, *B*, and *C* were prepared and placed in separate bottles and autoclaved for 20 minutes at 15 lbs. pressure and 120° C. Solutions *B* and *C* could be kept in the laboratory at room temperature for three or four weeks and would still be clear. However, it was found necessary to prepare Solution *A* every two weeks, at least, as molds seemed to grow in it. It could be kept in a refrigerator for a longer period of time without contamination.

In Table 2 are listed the ingredients for the three types of media used in this investigation. The control medium contained 10 g. of Fleischmann's fresh cake yeast (moist weight), which were mixed with Solution *A*, Solution *C*, Merck's reagent agar powder, Merck's reagent sucrose, and distilled water. This mixture, called Mixture I, was placed in a flask and autoclaved for 20 minutes at 15 lbs. pressure and 120° C. The 25 cc. of Solution *B* were autoclaved at the same time, but in a separate vial. Upon removal from the autoclave, Solution *B* was added to Mixture I and then the whole medium was boiled for one minute. For the experimental medium, a similar procedure was followed, with this difference. The 10 g. of yeast were mixed with the 25 cc. of distilled water and autoclaved for five hours at 15 lbs. pressure and 120° C., before they were added to the other constituents of Mixture I.

Keenan, Kline, Elvehjem, and Hart (1935) claimed that autoclaving yeast for five hours should inactivate thiamin but should not destroy vitamin B_2. However, until nicotinic acid and riboflavin were added to Mixture I, the larvae living on the experimental medium did not grow beyond the second instar. Therefore, these two substances were

TABLE 2

INGREDIENTS PER 100 CC. OF CULTURE MEDIUM

	Type of medium		
	Control	Experimental	Thiamin
Mixture I	1.5 g. agar 1 g. sucrose	same as control	same as control
autoclaved	25 cc. solution *A* 25 cc. solution *C*	medium	medium
20 min. at			
	10 g. yeast	10 g. yeast	same as
15 lbs.	25 cc. distilled water	25 cc. dist. water autoclaved 5 hours	experimental medium
pressure		14.3 mg. nicotinic acid	same as
& 120° C.		0.05 mg. riboflavin	control medium
Autoclaved 20 min. at 15 lbs. & 120° C.	25 cc. solution *B*	same as control medium	same as experimental medium
			0.08 mg. thiamin hydrochloride

added to Mixture I when it contained yeast autoclaved five hours. For the test or thiamin medium, the procedure was similar to that for the experimental medium. However, after Solution *B* was added to Mixture I and the whole medium was boiled for one minute, 0.1 cc. of a solution of Merck's thiamin hydrochloride in sterile distilled water was added so that there were 80 gamma per 100 cc. of culture medium.

After the medium was prepared, 3 cc. of it were placed, while still warm, into sterile 5 cc. Petri dishes and test tubes, by means of sterile pipettes. For each experiment, three sets, consisting of six Petri dishes and four test tubes, were prepared, one set containing the control medium, one, the experimental, and one, the thiamin medium. The test tubes were used to observe the late stages of larval development, the formation of pupae and emergence of adult flies, as it was found that they would keep sterile for a longer period than the Petri dishes. The dishes were used to observe the newly hatched larvae, since it was almost impossible to see them in the test tubes. Also, it was easier to remove the larvae quickly from the flat surface of the dishes, than it was to take them from the tubes.

Thin sheets of agar were spread on Pyralin spoons and covered with yeast suspension. Adult flies, about three days old, reared on

stock culture of banana agar, were fed fresh food for two or three days. Approximately 20 pairs of these flies were placed in small bottles containing the spoons upon which eggs were deposited during a two-hour period. These eggs were then scraped off the agar sheets with tiny glass spoons and sterilized in 80 per cent alcohol from 45 minutes to one hour. This treatment destroys any yeast cells which might be clinging to the eggs, but does not injure the embryo within. According to Baumberger (1919), yeast cells are not transmitted from the adult female to the eggs. Therefore, sterile larvae should have been produced by this treatment. Approximately 20 sterilized eggs were placed in each dish and tube containing the culture medium. The tubes and dishes were then placed in a sterile crystallizing dish and were kept in an incubator at $25^{\circ} \pm 0.2$ C. Forty-eight hours after the mean hour of the egg-laying period, two dishes of each type of medium were opened and a glucose-broth test was made for sterility. The larvae from these dishes were then taken out and killed in boiling water. This method, as described by Alpatov (1929), caused them to lie out straight without stretching or curling, and it was then possible to measure the body length with a compound Bausch and Lomb microscope, using a 48 mm. objective and a micrometer in a No. 10 ocular. Twenty-four hours later, when the larvae were 72 hours old, the same procedure was followed and likewise when they were 96 hours old. The tubes containing the glucose-broth tests were kept in an incubator at 37° C. and were observed 24 and 48 hours after they had been inoculated. Whenever the test indicated that a dish had been contaminated with live yeast or other micro-organisms, the measurements of larvae from that dish were discarded.

Results and Discussion

Observation of the results reveals two effects due to thiamin. First, an increase in the rate of development and second, an increase in the size of animals within certain stages. In Table 3 the percentage of animals in different stages of larval life is given. At 48 hours, the greater percentage of larvae grown on all three types of media was in the first instar. However, the control dishes held a larger percentage of larvae in the second instar than did the experimental dishes or the thiamin-containing dishes. At 72 hours, most of the larvae were in the second instar, but again the controls had a greater percentage in a more advanced stage. At 96 hours, the majority were in the third instar, but the control dishes had a greater

TABLE 3

PER CENT OF LARVAE IN EACH INSTAR AT 48, 72, AND 96 HOURS

Age	Instar	Type of medium: Control Number	Control Per cent	Experimental Number	Experimental Per cent	Thiamin Number	Thiamin Per cent
42 hrs.	(1)	209	63.7	246	97.6	289	96.7
	(2)	119	36.3	6	2.4	10	3.3
72 hrs.	(1)	6	2.9	3	1.4	8	3.1
	(2)	156	75.0	211	97.7	244	95.7
	(3)	46	22.1	2	0.9	3	1.2
96 hrs.	(1)	0	0	1	0.4	2	1.0
	(2)	7	2.9	48	20.5	19	9.6
	(3)	231	97.1	185	79.1	176	89.4

percentage than the thiamin dishes and these in turn, had a greater percentage than the experimental dishes.

With regard to the time of pupa formation, it was found that the control larvae began this process about 120 hours from egg-deposition and completed it 24 to 48 hours later. The larvae grown on the thiamin-containing medium did not begin to form pupae until 24 hours after the controls, and in the experimental tubes, the larval period was prolonged for another 24 hours, so that the experimental larvae did not begin to pupate until 48 hours after the controls.

Two conclusions can be drawn from these results. First, that some other factor or group of factors, other than thiamin, was destroyed by the five hours autoclaving of the yeast, since the animals receiving thiamin did not develop as quickly as the controls. Second, that thiamin seems to take its effect on the rate of growth after 72 hours of development, so that the larvae go more quickly into the third instar and also pupate earlier.

Since it is observed in Table 3 that a large proportion of larvae at each measuring period were in a certain instar, the measurements for

TABLE 4

AVERAGE LENGTH IN MILLIMETERS OF LARVAE IN EACH INSTAR

Age	Instar	Type of medium: Control	Experimental	Thiamin
48 hrs.	(1)	1.5 mm.	1.5	1.5
	(2)	2.025	2.1	2.1
72 hrs.	(1)	1.425	1.425	1.5
	(2)	3.0	2.625	2.775
	(3)	3.75	3.6	3.45
96 hrs.	(1)	none present	1.35	1.5
	(2)	3.075	2.925	3.0
	(3)	4.95	3.975	4.5

those instars have been italicized in Table 4. At 48 hours, the first instar larvae on all three types of media had the same average size, 1.5 mm. At 72 hours, the controls were 14.3 per cent larger than the experimentals, and the thiamin-treated animals were 5.7 per cent larger than the experimentals. At 96 hours, the controls were 24.5 per cent larger than the experimentals, and the larvae receiving thiamin were 13.2 per cent larger than the experimentals.

In Figure 1 are picture larvae representative of the sizes itali-

FIGURE 1

C E T are 48 hours old, first instar larvae of Drosophila.
C' E' T' are 72 hours old, second instar larvae.
C" E" T" are 96 hours old, third instar larvae.
The *C* larvae are control animals, the *E* larvae, experimental and the *T* larvae, thiamin animals.

cized in Table 4. This brings out more vividly the difference in body length due to thiamin, and also the difference in size due to some unknown factor in the control medium, which is lacking in the experimental and thiamin media. The results on the size therefore, seem to show the same phenomena as the results of the rate of growth. Some other factor or group of factors must be present in the control medium and lacking in the thiamin medium which increases the size of the larvae. However, the effect of thiamin can be clearly seen after the larvae are 72 hours old. There is a slight increase before the 72-hour measuring period, which is significant within the limits of

probable error, but the greater difference in size between the experimental and thiamin-treated larvae is evident at the 96-hour measuring period, when the animals are in the third instar.

This difference in size of larvae receiving thiamin and those growing on a medium lacking thiamin can perhaps be concerned with the rôle this vitamin plays in carbohydrate metabolism. This insect has three stages of larval life, and during the third instar it stores fat, glycogen and protein chiefly in the fat body. This process also occurs in the first and second instars, but it is increased in magnitude in the third in preparation for metamorphosis. The culture medium contained a high percentage of carbohydrate and protein and a rather low percentage of fat. However, the larvae are known to store most of the food material as fat and only a low proportion as glycogen and protein. The conversion of protein and carbohydrate to fat is therefore necessary. If thiamin plays a rôle in this conversion, it seems quite logical that its effect should be most evident when the larvae are so actively storing this food material.

The measurements made for this investigation are really only an indication of the degree of stretching or unfolding of the integument due to some kind of increase in mass inside the chitinous covering, and not to any actual increase in the exoskeleton itself. Therefore, any increase in the amount of stored material would cause an increase in the body length. It seems possible then, that thiamin increases the size of the third instar larvae of Drosophila by facilitating the carbohydrate metabolism and perhaps the fat storage. Since the experimental larvae did eventually pupate and emerge as adults, it can be concluded that thiamin is not strictly essential for growth of Drosophila, but it is influential both in the size of the larvae and their rate of development.

Acknowledgments

I wish to express my sincere acknowledgments to Professor Louise Palmer Wilson under whose direction this investigation was carried out.

Chemicals and facilities furnished by the Marine Experimental Station of the Lankenau Hospital Research Institute, and suggestions and criticisms of Dr. F. S. Hammett are also gratefully acknowledged.

I wish to thank Mr. Lloyd D. Wilson for the microphotograph included in this paper.

Summary

1. At 72 hours the body length of *Drosophila melanogaster* larvae, receiving thiamin, is 5.7 per cent longer than that of larvae lacking this vitamin. At 96 hours, thiamin treated larvae are 13.2 per cent longer than those deficient in vitamin B_1.

2. Thiamin in the culture medium of *Drosophila melanogaster* causes the larvae to develop at a more rapid rate so that the whole larval period is 24 hours shorter than that of animals lacking the vitamin.

3. Autoclaving Fleischmann's cake yeast in distilled water for five hours apparently destroys or inactivates, for use by *Drosophila melanogaster*, substances which are needed for normal growth other than vitamin B_1 riboflavin, and nicotinic acid.

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Growth, 1941, **5**, 27-52.

FURTHER GROWTH EXPERIMENTS WITH FLUORESCENT DYES*

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A. INTRODUCTION

It has been shown by the author (1940) that growth in plants can be increased with various—especially fluorescent—dyes. Many experiments have demonstrated that weak solutions cause accelerated growth and better development of the plant; concentrated solutions, on the other hand, inhibit growth, resulting in dwarfed plants.

In most cases, at the beginning of experimentation nine years ago, the dye used was Fluorescein alone brought into solution by the addition of bicarbonate of soda. Later, the Fluorescein was mixed with small amounts of iron and copper sulphate. These are the components of Photosensin.[1] Photosensin is used in a solution in which the concentration of Fluorescein varies between 1:100,000 and 1:2,000,000. The concentration of iron and copper sulphate was varied between 1:400,000 and 1:10,000,000. As much bicarbonate of soda is added as is required for dissolving the dye, not over 15 grams to 100 liters of water. It has been shown by the author (1940) that weak dye solutions may stimulate the development of "growth-promoting materials" in the plant, resulting in better and accelerated growth, while concentrated solutions may inhibit the development of "growth-promoting materials," or even destroy them, thus producing dwarf plants.

B. EXPERIMENTAL

The action of fluorescent dyes, especially of Photosensin, was made clear in the above-mentioned article. Recent experiments carried out by the writer in the Plant Physiology Department of the California Institute of Technology, in Pasadena, California, showed many interesting results from Photosensin treatment. They are presented in

*Received for publication on December 9, 1940.

[1]Instead of Photosensin powder, Photosensin tablets may be used. One tablet weighs 0.33 gram; 3 tablets 1 gram. One tablet in two gallons of water gives a solution of approximately 1:23,000, which is close to the generally used solution of 1:25,000. These quantities and concentrations are considered best in general use by L. M. McLaren Produce Inc., Yuma, Arizona, and therefore recommended on the Photosensin packages.

Tables 1 to 8 and in Figures 1 to 10. The soil was heavy adobe; all plants stood in full sunshine in the open.

1. *Experiment With Beets*

The seed was sown April 16th, 1940, in two rows of 5 m. each, which were fertilized once with ammonium sulphate. One row served as control and received water when the other row was given Photosensin. Treated and control rows were about 1.5 m. apart. Thirteen treatments were given 10 to 14 days apart, from April 15th to September 16th, by watering the row with one and one-third gallons of Photosensin solution. This solution was made weaker as the season advanced; from April 16th to June 29th, 0.66 g. (two tablets) Photosensin to four gallons of water (concentration 1:23,000); from June 29th to July 26th, 0.33 g. (one tablet) to four gallons (concentration 1:46,000); from July 26th to September 16th, 0.25 g. to four gallons (concentration 1:60,000). After each treatment, the soil of both rows was watered thoroughly with at least 2-3 gallons for each row. The harvest at September 24th is shown in Figure 1 and Table 1.

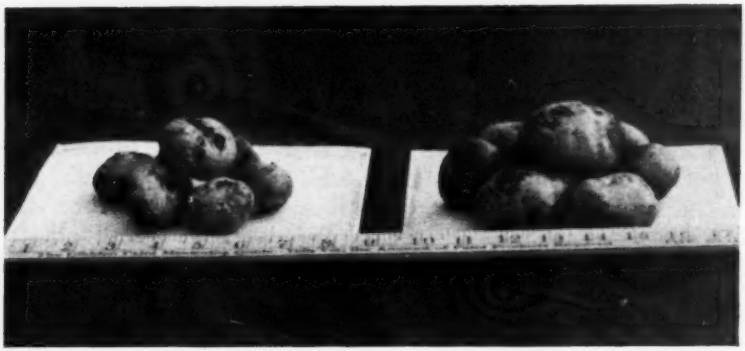

FIGURE 1

Beets, Sown April 16, 1940. Five months old. Left, untreated control; right, Photosensin treated.

TABLE 1

		Photosensin treated	Untreated
Number of plants		18	27
Average weight of single beet	(with top	41.8 grams	32.9 grams
	(without top	35.1 grams	25.9 grams
Increase in weight of crop by Photosensin treatment		27% with tops 35% without tops	

2. *Experiment with Marigolds*

Seed was sown April 16, 1940, and treated in the same manner as the beets. The foliage of treated marigolds was sturdier, the blooms larger and fuller (see Figure 2), showing a remarkable superiority in

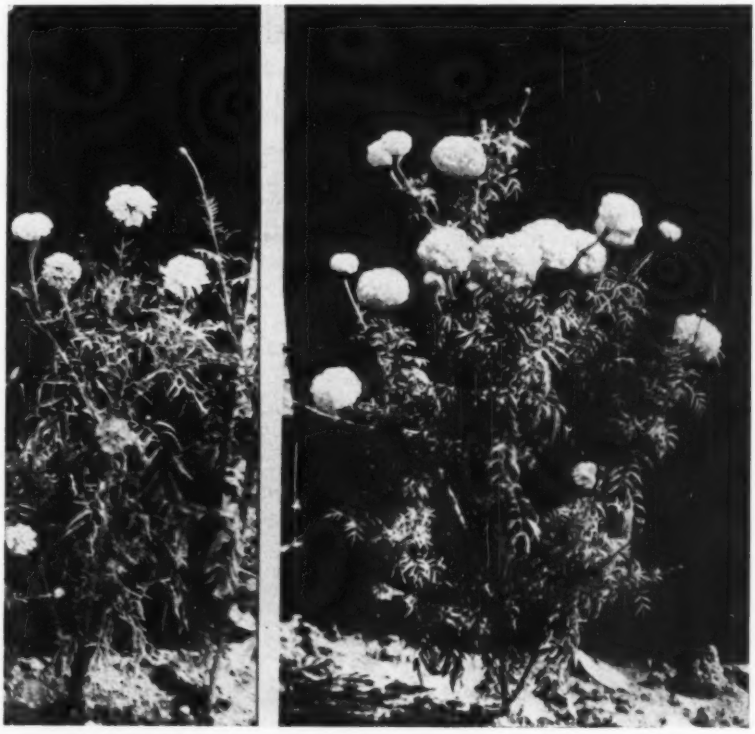

FIGURE 2

Marigolds. Sown April 16, 1940. Four months old. Left, untreated control; right, Photosensin-treated plant.

quality. The number of large blooms was greater in treated plants. All flowers were cut September 3rd; the second flowering showed the same difference in quantity and quality of blooms as first flowering. The treated blooms lasted longer. The number of blooms is shown in Table 4.

3. *Experiment with Chrysanthemums*

Three-months-old untreated plants were transplanted July 9th, 1940, into two plots of two square meters each. One plot served as control and received water, while the other plot was given Photosensin. Treated and control plots were about 1.5 m. apart. Nine treatments were given 10-14 days apart, from July 9th to October 11th, by watering the treated plot with two gallons of Photosensin solution. This solution was changed as the season advanced. For the first treatment, two tablets of Photosensin (0.66 gram) were dissolved in four gallons of water (concentration 1:23,000); for the following three treatments, 0.25 gram of Photosensin powder to four gallons of water (concentration 1:60,000); thereafter, 0.50 gram Photosensin powder to four gallons of water (concentration 1:30,000). Immediately after each Photosensin treatment the soil of both plots was thoroughly watered with at least 2-3 gallons per sq. meter. The harvest at October 16th is shown in Table 2.

TABLE 2

		Photosensin treated	Untreated control	Increase in blooms by Photosensin treatment
Number of plants		19	18	
Total number of blooms		600	344	
Number of blooms per plant	Highest	51	27	24
	Lowest	18	10	8
	Average	31.6	19.1	65.4%

The average number of blooms per plant has been evaluated statistically[2] and the standard error calculated according to the formula:

$$e = \sqrt{\frac{\Sigma d^2}{n\ (n\text{-}1)}}$$

where d = deviation from the mean of single observations, and n = number of plants. The *Standard Error* of the treated plant has been found to be: $\pm$ 1.9, and the *Standard Error* of the untreated plant has been found to be $\pm$1.3.

$\text{Mean}_{\text{treated}}$: 31.6 ± 1.9

$\text{Mean}_{\text{control}}$: 19.1 ± 1.3

The difference between treated and control (untreated) chrysanthe-

[2]The other experiments have not been evaluated statistically on account of too low observation numbers; the results are based on arithmetical calculation. The improvement also is plainly visible in the pictures taken.

mums is *highly significant*, as the probability of the occurrence of the "Mean Treated" in a normal untreated population is less than 1:1000. On November 5th all chrysanthemums were taken out of the ground; tops and roots were separated and weighed.

TABLE 3

INCREASE IN TOPS AND ROOTS OF CHRYSANTHEMUMS BY PHOTOSENSIN TREATMENT

	Number of plants	Weight of top		Increase by Photosensin treatment	Weight of root		Increase by Photosensin treatment
		Total grams	per Plant grams		Total grams	per Plant grams	
Treated	15	369	24.6	60%	35	2.33	67.7%
Untreated	14	214	15.3		19.5	1.39	

4. *Experiment with Asters*

On July 9, 1940, three-months-old untreated plants were transplanted and treated in the same manner as chrysanthemums. The treated plants bore more blooms, and their quality improved. On November 5th all plants were taken out of the ground, their roots separated from the tops and weighed. The treated plants had larger roots. The total weight of roots of 13 treated plants was 41.7 grams; of 11 control plants 22.2 grams. The increase in weight of roots per plant was 60 per cent. The untreated asters were dying when treated asters were still alive. The results of flowering are shown in Table 4.

5. *Experiment with Millet*

The seed was sown July 9th, 1940, in two plots of 1 sq. meter each. One plot served as control and received water, while the other plot was given Photosensin. On July 19th some new seed was sown in a ½ sq. meter area. Three treatments were given by watering with 1.5 liters of a Photosensin solution. For the first treatment given July 10th, the solution was prepared by dissolving one tablet (0.33 gram) of Photosensin to two gallons of water (concentration 1:23,000), and further diluted to get a concentration of 1:25,000; for second and third treatments (July 22nd and August 6th) a weaker solution was made by dissolving 0.25 gram of Photosensin powder to two gallons of water, and further diluted to get a concentration of 1:35,000. Treated and untreated plots were watered with 1.5 gallons per sq. meter immediately after the Photosensin treatment. The difference between treated and control millet was evident within 14 days; it became very remarkable within 28 days when the foliage of treated

TABLE 4

EXPERIMENTS CARRIED OUT AT PASADENA WITH PHOTOSENSIN, MARCH TO OCTOBER 1940

Plant	Sown	Record taken		Concentration of Photosensin solution used	Number of treatment	Number of plants	Number of blooms Total	Number of blooms per Plant	Crop in grams Total	Crop in grams per Plant	Increase by Photosensin treatment
Beet	Apr. 16	Sep. 24	Treated	1:23,000 1:46,000 1:60,000	13	18	—	—	632	35.1	35.5%
			Control	—	—	27	—	—	700	25.9	
Marigold 1st flowering	Apr. 16	Aug. 21	Treated	Same as beets	11	15	77	5.1	—	—	32.4%
			Control	—	—	26	98	3.7			
Marigold 2nd flowering	Apr. 16	Sep. 24	Treated	Same as beets	13	14	94	6.7	—	—	45%
			Control	—	—	23	106	4.6			
Radish	Mar. 28	May 8	Treated	1:23,000	4	510	—	—	Top 861.9 Root 1055	1.69 2.07	11.9% —
			Control	—	—	550	—	—	Top 830 Root 1133	1.51 2.06	
Zinnia	Jul. 9	Nov. 1	Treated	1:46,000 1:60,000	9	20	81	4.1	—	—	
			Control	—	—	19	84	4.4			
Chrysanthemum	Jul. 9	Oct. 16	Treated	1:23,000 1:60,000 1:30,000	9	19	600	31.6	—	—	65.4%
			Control	—	—	18	344	19.1			
Aster	Jul. 9	Oct. 24	Treated	Same as chrysanthemums	9	13	197	15.1	—	—	46%
			Control		—	12	124	10.3			

plants was much sturdier, and the difference in height was almost 100 per cent, as shown in Figures 3 and 4. Harvested control and

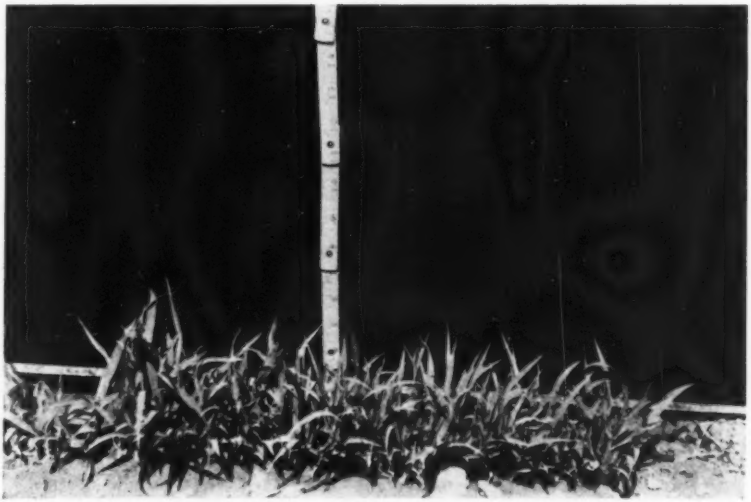

FIGURE 3
Millet untreated. Sown July 9, 1940, 28 days old.

FIGURE 4
Millet treated with Photosensin. Sown July 9, 1940, 28 days old.

TABLE 4

EXPERIMENTS CARRIED OUT AT PASADENA WITH PHOTOSENSIN, MARCH TO OCTOBER 1940

Plant	Sown	Record taken		Concentration of Photosensin solution used	Number of treatment	Number of plants	Number of blooms Total	Number of blooms per Plant	Crop in grams Total	Crop in grams per Plant	Increase by Photosensin treatment
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			Control	—	—	27	—	—	700	25.9	
Marigold 1st flowering	Apr. 16	Aug. 21	Treated	Same as beets	11	15	77	5.1	—	—	32.4%
			Control	—	—	26	98	3.7			
Marigold 2nd flowering	Apr. 16	Sep. 24	Treated	Same as beets	13	14	94	6.7	—	—	45%
			Control	—	—	23	106	4.6			
Radish	Mar. 28	May 8	Treated	1:23,000	4	510	—	—	Top 861.9 Root 1055	1.69 2.07	11.9% —
			Control	—	—	550	—	—	Top 830 Root 1133	1.51 2.06	
Zinnia	Jul. 9	Nov. 1	Treated	1:46,000 1:60,000	9	20	81	4.1	—	—	
			Control	—	—	19	84	4.4			
Chrysanthemum	Jul. 9	Oct. 16	Treated	1:23,000 1:60,000 1:30,000	9	19	600	31.6	—	—	65.4%
			Control	—	—	18	344	19.1			
Aster	Jul. 9	Oct. 24	Treated	Same as chrysanthemums	9	13	197	15.1	—	—	46%
			Control		—	12	124	10.3			

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FIGURE 3
Millet untreated. Sown July 9, 1940, 28 days old.

FIGURE 4
Millet treated with Photosensin. Sown July 9, 1940, 28 days old.

FIGURE 5
Millet from Figure 3 (untreated), two months old.

treated millet have been divided into four groups according to height and quality of plants. Plants in Groups 1 and 2 are superior, normal in Group 3, and small in Group 4. The harvest of ½ square meter area each at September 5th and 24th is shown in Table 5. Figures 5 and 6 show millet two months after sowing, and Figure 7 illustrates difference in quality of treated and control millet.

FIGURE 6
Millet from Figure 4 (treated), two months old.

6. *Experiment with Radishes*

Seed was sown March 28th, 1940, in two plots ¾ sq. meters each; treated four times with one gallon of a solution prepared by dissolving one tablet (0.33 gram) Photosensin in two gallons of water (con-

TABLE 5

PHOTOSENSIN EXPERIMENT WITH MILLET CARRIED OUT AT PASADENA

		Treated	Control *a*	Control *b*
Sown		July 9	July 9	July 19
Harvested		Sept. 5	Sept. 5	Sept. 24
Concentration of Photosensin used	1.	1:25,000	0	0
	2.	1:35,000	0	0
	3.	1:35,000	0	0
Plant groups according to height and quality: number of plants per group	giant	34	24	11
	large	62	57	45
	normal	88	166	101
	small	64	399	113
	Total	248	646	270
Grain groups according to size: Grams of grain in each group	giant	47	12.5	7
	large	67	29	19.5
	normal	29	47.5	21
	small	5.5	23.5	6
	Total	148.5	112.5	53.5
Grain per plant, grams		0.59	0.18	0.19
Increase by Photosensin in per cent		Over 100	—	—

centration 1:23,000). Harvested at May 8th. Results are shown in Table 4. The sowing was overcrowded, the weather chilly.

7. *Experiment with Zinnias*

Three-months-old untreated plants were transplanted July 9th, 1940, into two plots of 2 sq. meters each. One plot served as control and received water, while the other plot was given Photosensin. Nine treatments were given 10-14 days apart, from July 9th to October 16th, by watering one plot with two gallons of a Photosensin solution. From July 9th to July 20th the solution used was made by dissolving one tablet (0.33 gram) of Photosensin in four gallons of water (concentration 1:46,000). After July 26th a weaker solution was used by dissolving 0.25 gram of Photosensin in four gallons of water (concentration 1:60,000). Large flowers were cut from August 23rd on. All flowers were counted. The number of blooms is shown in Table 4.

8. *Experiment with Peanuts and Soya Beans*

Peanuts and soya beans were sown in two beds July 10th, 1940, 1 sq. meter of each plant in each bed. One bed was treated with a Photosensin solution every 10 days, the other remained untreated. The Photosensin solution used was the same as for zinnias. Treated

FIGURE 7

Millet. Illustrating improvement in quality after Photosensin treatment. Two-months-old plants. Left: untreated control; right: Photosensin-treated millet.

peanuts and soya-beans germinated earlier and better, and showed improvement in development as early as 2-6 weeks. However, due to the advanced season, this experiment could not be followed to maturity.

C. Analysis of the Experiments

It can be stated that, where Photosensin has proved effective, not only a mere increase in flowering and crop could be observed, but also an improvement in quality. Stems and foliage of treated plants looked sturdier. The blooms of marigolds and asters, for instance, were fuller; the treated plants had a greater percentage of large blooms than the untreated ones. The treated flowers lived longer than the controls. An increase in the weight of roots could also be stated (beets, asters, chrysanthemums).

In the experiment with *millet,* one of the two controls (*a*) showed overcrowded sowing, but the result in weight and quality hardly diff-

TABLE 6

DISTRIBUTION OF GRAIN WEIGHT ACCORDING TO QUALITY IN TREATED AND CONTROL MILLET

	Group 1 Giant-size	Group 2 Large-size	Group 3 Normal-size	Group 4 Small-size
Treated millet	31.7%	45%	19.6%	3.7%
Control (*a*)	11.1%	25.9%	42.3%	20.7%
Control (*b*)	13%	36.5%	39.2%	11.2%

ered from control (*b*) where sowing was normal. Table 6 shows how the total weight of grains in treated and control plants is distributed among the various groups. This points to quality. The figures are calculated from Table 5 in multiplying the weight of grains in each group by 100 and dividing by the corresponding total weight of all four groups.

As evident from this statement, in treated millet Groups 1 and 2 (superior quality) prevail, whereas only 3.7 per cent of the total weight belongs in Group 4 which for practical purposes is worthless. The controls, on the contrary, contain less of best quality grains and considerably more of the poor quality. The difference showed also between treated and untreated plants; in the treated, stems were longer (height of treated plants in the two largest groups 85-110 cms.; in controls, 60-90 cms.). Ears and grains larger as may be seen in photograph, Figure 7.

In the case of radishes and zinnias, the Photosensin solution used was apparently an improper one. Crowded sowing, and the cold April temperature of 1940, might also have influenced the development of radishes.

Attention may here be drawn to some previous publications by the same writer where under proper soil, planting, and weather conditions, with the aid of a properly chosen dye solution, results of Photosensin treatment in radishes and zinnias have been very remarkable.

As may be clearly seen from Tables 4 and 5, the concentration of the Photosensin solution had to be changed during the course of most of the experiments. This was due to the fact that conditions for treatment under these special circumstances were not known before the beginning of the experimentation. For weeks, treated marigolds and beets were only slightly better than the control plants. It must therefore be supposed that the concentration of the Photosensin solution was not optimal for these plants, and hence the application of weaker solutions seemed indicated. Similar reasons were responsible for other changes in the concentration of the solution. These differences can be overcome by the "systematic method" explained later.

Whenever Photosensin is first applied under new conditions, great attention must be be given to the soil itself. In the garden where these experiments were carried out, the soil had no humus layer whatsoever, and fertilization was tried on one experimental plot and in one instance. Photosensin treatment usually needs a very well fertilized soil.

1. *Dwarfing Experiments with Concentrated Dyes*

Fluorescent dyes, when applied in strong concentrations, will not stimulate growth; they will, on the contrary, inhibit growth and produce dwarf plants (Sellei, 1940).

2. *The Use of Concentrated Fluorescent Dyes for Dwarfing*

For dwarfing, Dimin is used, the components of which are also Fluorescein with small amounts of iron and copper sulphate, and bicarbonate of soda, but the concentration in which this dye is used is at least 100 times stronger than that of the strongest Photosensin solution.

Dimin for dwarfing experiments should be applied only to pots, as it is not advisable to use a highly concentrated fluorescent dye in open ground where it may affect the soil to a degree of inhibiting growth longer than desirable.

To 4-inch pots containing one pound of soil one or two grams of Dimin were added per pot. With increasing quantities of Dimin used, gradually smaller plants with correspondingly smaller stem, leaf and fruit growth are obtained. Treatment was applied only once, immediately before the seeds were placed in the pots. This applies to young plants also. The effect of Dimin was more spectacular when used on seeds. The pots were watered immediately after the Dimin treatment. Later on they were watered as usual. The controls were raised and kept under identical conditions, care being exercised not to place them too close for contamination with Dimin.[3]

3. *Dwarfing Experiments with Dimin*

Carried out with *Castor Beans, Marigolds and Zinnias*

Seed set: in 4-inch pots (1 lb. of soil per pot).
Date of setting: Jan. 27, 1940.
Treated with Dimin: Jan. 27, 1940.
Number of Treatments: One.

[3]To obtain still more effective dwarfing, Dimin may be used at 6-8 week intervals, two or three times to a plant in the course of a season, and for the third treatment only half of the original amount should be applied.

Series I: 1 gram Dimin per pot.
Series II: 2 grams Dimin per pot.
Series III: Control—No Dimin.
Record taken March 27, 1940.

TABLE 7

HEIGHT OF INDIVIDUAL DWARFED AND CONTROL PLANTS (IN CM.)

		Castor beans	Marigolds	Zinnias
Series I; 1 g. Dimin	Single plants	8 7.5	6, 5, 7, 4	5.5, 4.5, 4
	Average	7.8	5.5	4.8
Series II; 2 g. Dimin	Single plants	7, 7, 5.5	6, 5, 3, 5	2, 1
	Average	5.6	4.8	1.5
Control; no Dimin	Single plants	15, 16, 13	22, 17, 8.5	13.5, 11, 10
	Average	14.6	15.8	11.3
Control higher than average	Series I	87%	187%	135%
	Series II	160%	250%	over 250%

The plants in Series 1 and 2 were considerably dwarfed in comparison with the control plants. Foliage of treated plants was smaller and also blossoms and blooms of treated marigolds and zinnias—visible in 3½ to 4 months after sowing—were of small size; however, the dwarfed plants were normally developed and healthy. The experi-

FIGURE 8

Zinnias, dwarfed by Dimin. Sown Jan. 27, 1940. Six weeks old. Left, untreated control; center, treated with 1 gram Dimin; right, treated with 2 grams Dimin.

FIGURE 9

Marigolds, dwarfing experiment. Sown Jan. 27, 1940, 4½ months old. Left, untreated control plant; right, dwarfed plant.

ment was finished before either of the castor bean plants, control or treated, could bear flowers.

4. *Dwarfing Experiment with Dimin Carried Out with Corn Plants*

Seed sown: March 17, 1940. Four days old normal corn seedlings

were transplanted into 6-inch pots each containing 4 lbs. of soil, one plant to each pot. Treated with Dimin March 21st. Series *A*, two grams of Dimin per pot; Series *B*, control-untreated plants. Records were taken April 25th and July 31st.

TABLE 8

	Height of plant with longest leaf		Height of plant itself	Length of fruit
	April 25	July 31	July 31	July 31
Series *A*: 2 g. Dimin Dwarfed Plants:				
No. 1	46.5 cm.	47 cm.	30 cm.	9 cm.
No. 2	50 cm.	65 cm.	44 cm.	13 cm.
No. 3	52 cm.	75 cm.	57 cm.	12 cm.
Average	49.5 cm.	62.3 cm.	43.7 cm.	11.3 cm.
Series *B*: no Dimin				
Control No. 1	56 cm.	105 cm.	79 cm.	16 cm.
Control No. 2	69 cm.	died	— cm.	— cm.
Control No. 3	71 cm.	106 cm.	78 cm.	19 cm.
Average	65.3 cm.	105.5 cm.	78.5 cm.	17.5 cm.
Control larger than dwarfed	31.9%	69%	80%	55%

D. Discussion

Generally speaking, for accelerated growth and better development, treatment with a Photosensin solution of about 1:25,000 has proved best for most plants, especially for flowers; however, this cannot be considered as a set rule because flowers, as well as other plants, may call for different solutions under altered conditions. Only systematic experiments can lead to the recognition of the proper amount of dyestuff to be used in a given case. Seeds, as well as young plants, should be planted in their proper sowing period only. When sown earlier or later, good results cannot be expected even when treated with Photosensin systematically. Photosensin treatment must always be in accordance with the plant's nature and its light-sensitive and Photosensin-sensitive period.

In colder seasons or climates, possibly more Photosensin is needed for the same plant. In other words, preferably weak concentrations should be used during the hot summer months, while in the spring, stronger concentrations will give better results. For this reason, in Arizona and Southern California, large quantities of water were applied immediately after each Photosensin treatment, as mentioned in describing experiments in Pasadena, California. Soil conditions also

FIGURE 10

Dwarfing experiment with corn. Left, untreated control; right, dwarfed plant. Both plants bear fruit.

had to be considered. In Central Europe and in England, where the climate is much cooler, the Photosensin treatment was usually sufficient without any additional water. In Hungary and England, for most plants five liters of a 1:20,000 or 1:30,000 solution (etc.) to a

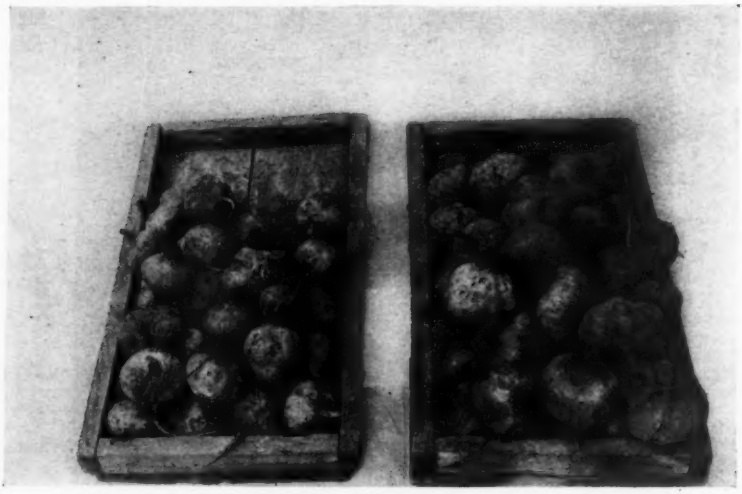

FIGURE 11

Beets. Sown May 2, 1938, 4½ months old. Left, untreated control; right, treated with Photosensin.

square meter surface proved to be just right without additional water.

The good results obtained by the use of highly diluted solutions, and the advantage of additional watering immediately after the Photosensin treatment in the warmer climate, suggest that Photosensin can exert its full action only when light, temperature, and water are properly balanced. No doubt Photosensin treatments require not only the proper amount of light energy, but also the optimal temperature which, in open ground experiments, is not available except in the summer and fall seasons. It can be said, therefore, that open ground experiments carried out during the warm season show better results than such experiments tried on the same plants and soil during the cold season, although from an empirical point of view, it might have been supposed that in the colder season a stronger Photosensin solution would compensate for the lack of heat. As a matter of fact, best results in open ground experimentation with Photosensin, under any kind of climatic conditions, were always obtained in the late spring, summer, and early fall periods (from the beginning of May until the end of September).

If the circumstances are not favorable, the plant may not respond to Photosensin treatment. Failures may be caused by the use of too

highly diluted solutions, or of too concentrated dyes, by cold weather, lack of sufficient light, too low a temperature, poor and unfertilized soil, sowing out of season (i.e., fall wheat sown in spring), too close planting of treated and control plants, and insufficient watering. As an example, the radish experiment might be mentioned. Several experiments have shown quite unusual effects of treatment on radishes. In Pasadena, however, where treatment was administered during chilly weather in open ground, with a presumably too concentrated solution, treatment proved unsuccessful.

The proper conditions, combined with a systematic method of treatment, can certainly show how fluorescent dyes work effectively on plants.

If conditions are correct for experimentations, Photosensin treatment will result in: acceleration in the development of plants, sturdier roots, stems and foliage, and a more abundant flowering and crop. The increase varies from 25 per cent to over 100 per cent. There will also be an improvement in quality.

Properly used, Photosensin also increases the vitality of plants. While untreated plants are dying off, Photosensin-treated plants, kept under similar conditions, are fresh, and still bear flowers. Cut flowers also last longer when placed in a Photosensin solution, and treated vegetables stay fresh, keeping their full flavor for a longer period after harvesting.

Recent experiments carried out by Louis S. Zopf (1940) at the State University of Iowa have shown that top growth response of castor beans (*Ricinus communis*) to the combined use of Vitamins B_1 and C can be further increased with Photosensin.

The method of applying fluorescent dyes for increasing flowering and crop might be improved by further experimentation. How this growth stimulation is effected, calls for still further investigation, but the writer believes that his suggestion to promote plant growth by means of highly diluted fluorescent dyes, a study on which he worked over a nine-year period, is definitely proved by the experiments carried out in Pasadena.

E. Determination of the Optimal Dye Concentration

The previous article (1940) explains how different results can be obtained by using various concentrations of a given dye solution on the same plant. (Experiment with "Non-Pareil" beans and Darwin tulips in pots; and Green Nordstern Beans in open ground.) Such

experiments, if carried out systematically, will indicate which dye solution is most effective for the given plant under definite circumstances.

1. *The Systematic Method*

a. Photosensin treatment in pots. When treating plants with Photosensin, either in pots or in open ground, the soil of treated and control plants must be thoroughly fertilized. During a longer planting period it is advisable to renew fertilization.

The above-mentioned experiment with Darwin tulips illustrated the action of various concentrations. Each pot contained 300 grams of well fertilized soil, and all pots received 30 per cent of the Photosensin solution (calculated on the weight of soil), i.e., 90 cc. per pot at each treatment, beginning with a solution of one gram Photosensin powder to 10 liters of water, and gradually increasing the amount of water until a solution of one gram Photosensin to 20, 25, and 30 liters was reached. It was shown that best results with these tulips were obtained by using a concentration of 1:25,000. Generally, this kind of experiment is carried out in the following way.

Several series containing 5-10 pots each of the same size are used. All the pots are filled with the same amount of potting soil. Two of the series represent controls without any Photosensin; the others get the same amount of solution, but containing different concentrations of the dye, graded from 1:10,000 to 1:30,000. The amount of the solution may be either 10, 20, or 30 per cent of the weight of the soil in the pot. The amount as well as the concentration has to be tested for the given plant. In many cases 20 per cent of the solution proved best.

Another method is treatment with just one concentration, for instance 1:25,000 or 1:20,000, applying various amounts of this solution, e.g., several series of 5-inch pots (with 2 lbs. of soil) were treated, one series with 50 cc., others with 100 cc., and 200 cc. of the same solution, water being added in each case to make the total amount of water given equal 200 cc. The above methods are usually recommended.

For the purpose of simplifying the application of dyes in pot experiments, Photosensin powder was mixed with an indifferent agent such as milk-sugar so that 0.5 gram contained as much Photosensin as 100 cc. of a 1:20,000 solution, namely 5 mg.; ½, 1, 2, and 3 powders may be given to different experimental series. Treatment must be followed by watering with 100 cc. of water to each pot, controls get same amount of water.

In all pot experiments, treatment is to be repeated each 10-14 days.

b. Photosensin treatment in open ground. Open ground also requires systematic experimentation to determine what amount of Photosensin should be used for a certain plant under certain circumstances.

It is necessary that an area be treated several times with the same amount of Photosensin powder regardless of how much water is required. To determine what amount of Photosensin will give best results on a certain area in open ground, a systematic routine must be established. E.g., a number of experimental plots of the same size are treated with a Photosensin solution of: (*a*) different concentrations in equal amounts; (*b*) the same concentration in varied quantities; (*c*) the same concentration in the same quantities but immediately followed by varying additional watering.

Experiments carried out in accordance with this suggestion have been published in previous articles by the same writer; here, only one example for each group should be mentioned (Tables 9-11).

TABLE 9

EXPERIMENT WITH POTATOES (Abingdon, England 1937, H. G. Longford)

Concentration of Photosensin solution used	Number of plants	Weight of crop, total	Weight of crop per plant (average)
5 liters of:			
1: 10,000 (*)	5	8.563	1.713
1: 12,500	6	13.250	2.208
1: 15.000	5	8.313	1.663
1: 17,500	6	10.500	1.750
1: 20,000	6	13.938	2.323
1: 22,500	6	9.375	1.563
1: 25,000	5	11.500	2.300
1: 35,000	5	12.750	2.550
1: 40,000	4	10.438	2.609
1: 50,000	4	6.500	1.625
1: 70,000	5	11.000	2.200
Control:	12	18.875	1.572

Optimum results with solution 1:40,000, e.g., one gram Photosensin to 40 l. of water; increase in weight 66 per cent.

*By dissolving one gram of Photosensin powder to 10 l. of water. The other concentrations were obtained in a similar way.

The main factor in all Photosensin experiments obviously is the amount of Photosensin powder used. However, the quantity of water in which this powder has been dissolved is not without importance. Many experiments show the advantage of dissolving the powders in very large amounts of water. The action of Photosensin is more intensive when applied in highly diluted solutions. The relation be-

TABLE 10

EXPERIMENT WITH BEETS

(Municipal Gardeners' Training School, Budapest, Hungary. Dr. Joseph Sellei.)

Concentration of solution used: 1:27,500, i.e. 1 gram Photosensin to 27.5 l. of water	Weight of crop on 10 square meters, grams	Weight of crop per square meter, grams
1.) 5 l. per sq. meter	6120	612
2.) 8 l. per sq. meter	4570	457
3.) Control (untreated) *a.*	3260	326 (Mean 340
4.) Control (untreated) *b.*	3530	353 grams)

Sown May 2, 1938; harvested Sept. 20, 1938.
Best results: 5l. solution per sq. meter. Increase 80 per cent.

TABLE 11

EXPERIMENT WITH MILLET

(Municipal Gardeners' Training School, Budapest, Hungary, Dr. Joseph Sellei.)

Concentration of Photosensin solution	Amount of solution per sq. meter	Water added per sq. meter	Height of plant	Length of ears
1.) 1:27,500*	1.5 l.	1.5 l.	88 cm.	19½ cm.
2.) 1:27,500	1.5 l.	5 l.	96 cm.	21 cm.
3.) 1:27,500	1.5 l.	8 l.	91 cm.	20 cm.
Control: plain water, as much as in treated			72 cm.	18 cm.

4 sq. meter plots each.
Sown June 19, 1938—Record taken Sept. 8, 1938.
Best results obtained with 1.5 l. Photosensin solution + 5 l. of water; increase in height: 33.3 per cent.

*1 gram Photosensin to 27½ l. of water.

tween concentration and distribution of the dye must be considered to be similar to the well known fact that one drop of aniline yellow dissolved in a 3-4 liter basin of water will be fully absorbed by a greasless cotton thread. The plant root affected by highly diluted dye solutions, i.e., Photosensin solution, acts in a similar way.

Instead of Photosensin solutions, gradually increasing amounts of Photosensin powder can be used to a series of areas of uniform size. (The powder used for open ground experiments is the plain Photosensin powder itself, not mixed with milk sugar as described in pot experiments.) For instance, experiments may be carried out by applying the following amounts of Photosensin per square yard: 0.7 grain*, 1.0 grain, 1.5 grains, 2.5 grains, 3.5 grains.

*Note that in this experiment the figures are in grains and square yards, while all previous experiments were recorded in grams and square meters.

The powder should be dissolved in 1-2 gallons or more of water depending on the quality of soil, the amount of moisture it requires, temperature, climatic conditions, sunshine, etc. If large areas are concerned, 0.7 grain per 1 sq. yd. will make about 200 grams Photosensin powder per acre.

In large scale experiments, the powder should be added to the irrigation water, if possible.

In plants which have been dwarfed by natural selection taking place over a period of several years, Photosensin treatment will not produce large blooms or foliage; pompom flowers will not change character after treatment.

F. Photosensin Applied in High Dilution

The Photosensin treatment of plants can be applied to large as well as small areas. Small areas should be treated (*a*) with tablets (three tablets making one gram) and water by sprinkler; (*b*) with powder, and also watered by sprinkler. Large areas should be treated with powder, (*a*) by the irrigation method; (*b*) by simple watering.

1. *For Irrigation*

The powder is first dissolved in a barrel of water; after being transferred into a syphon, this solution is instilled drop by drop into the irrigation water, which is distributed over the whole area. According to the field, ½, ¾, 1, 1.5, 2 kilograms of the powder should be used per acre. The exact amount must always be found first by the systematic method already described.

When making trial tests with Photosensin, check plots should always be arranged with the use of water instead of Photosensin. Between the different treated areas, as well as between treated and control plots, a space of 20-30 ft. should be provided, as otherwise the results cannot be separated. (No planting should be done in these spaces.) An experimental field may properly be divided as follows:

Control—½ kg. PSS—¾ kg. PSS. Control—1 kg. PSS—1.5 kg. PSS 2 kg. PSS—Control. (PSS = Photosensin.)

After each two or three treated plots, a control plot should be inserted. As the quality of the soil changes, it is necessary to make several control plots, all of which should be at a proper distance from the treated plots. Plots treated with Photosensin should not be used as controls in a subsequent experiment, as the soil may retain some dye which might affect such experiments for some weeks.

In late fall and early spring, the following amounts have given best results for certain plants in Yuma, Arizona:

Lettuce	1—1.5 kg. Photosensin powder to the acre
Carrots	1 kg. Photosensin powder to the acre
Sugar Beets	¾ kg. Photosensin powder to the acre

This treatment was repeated 3-4 times at intervals of about 3-4 weeks. Under other circumstances, other amounts of Photosensin may apply for the same plants.

2. *When Should the Photosensin Treatment Begin?*

This depends upon the plant. With plants which grow quickly, 2-5 days after sowing; with plants which grow slowly, only 2-3 weeks after sowing. Open ground treatment should be applied to seeds following sowing, and to young 2-3-months-old plants. When plants already 6-8-weeks-old are placed outdoors, these must immediately be treated with Photosensin after placing them in the garden soil, and the treatment should be repeated at intervals of 10-14 days. In some cases, Photosensin treatment of older plants also has proved successful. Pecan trees, for instance, showed improvement in development after Photosensin treatment. This kind of treatment, however, calls for further experimentation.

3. *How Frequently Should Plants be Treated?*

Vegetable seeds should be treated 3-5 times; flowers 9-10 times and even more frequently. One application is insufficient. Between treatments, particularly for flowers in the open, as well as in pots, sprinkling with water as usual is necessary. Garden and pot plants are usually treated each 10-14 days; field plants 3-4 times at intervals of 3-4 weeks.

G. Estimate of Amount of Water to be Used for Experiments Carried Out in Small Areas

It must be determined, first, *how much water* one square meter of soil can absorb. In Arizona and Southern California, for instance, 12 to 15 liters may be absorbed by 1 sq. meter of soil surface in dry summer weather. The rule is to saturate the soil thoroughly with water. That may be 15 liters or only 2-3 liters per square meter (according to soil and climatic conditions). The water must be well absorbed by the soil.

After rains, if the soil is wet and the season more advanced, the

soil should again be tested to ascertain how much water it is able to absorb. Only as much water should be added after each Photosensin treatment as the soil requires to become thoroughly moist. Too little or too much water is equally wrong for Photosensin treatment. The proper Photosensin solution is one of the conditions for the success of the treatment.

H. Summary

1. The effect caused by fluorescent dyes on plants depends mainly on the concentration and on the amounts used.

2. Highly diluted fluorescent dyes when applied under proper conditions of soil, weather, light and planting, in proper concentrations, stimulate plant growth: root, foliage, flowers and crop will be sturdier and more abundant.

3. In most of the cases, treated plants will show improvement of quality.

4. Highly diluted fluorescent dyes can also show favorable results when used together with other plant stimulants.

5. Concentrated dyes inhibit growth without having a toxic effect, as the dwarfed plants bear flowers and fruit.

6. Methods have been worked out to determine the optimal dye concentration under different conditions of plant growth.

The author is indebted to the California Institute of Technology, Pasadena, California, for making available facilities for the preceding experiments, and to Mr. Th. Barrett for the daily care of the plants.

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Growth, 1941, **5**, 53-59.

THE FORM OF PIGMENTATION MARKINGS*

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A. INTRODUCTION

The elements which make up the coloration patterns of animals frequently have their boundaries in the form of arcs of circles. This is especially noticeable where the markings are sharply delineated, as in Lepidoptera and birds, but molluscs, fishes, and mammals illustrate the same condition with such frequency as to suggest the generalization that the arc of a circle is a fundamental element in coloration pattern. A series of examples is shown in the accompanying figures.

It will be noted that the patch may be bounded by: (*a*) the circumferences of two circles, one of which is contained, either concentrically (Figure 2*C*: ringed seal), or excentrically (ocellus in peacock)

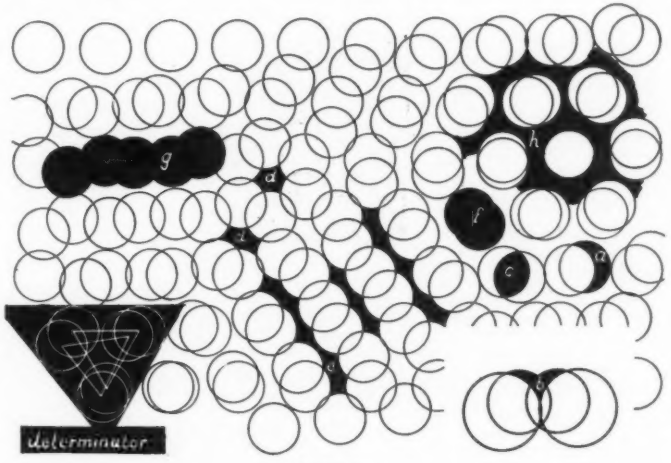

FIGURE 1

GEOMETRICAL DIFFERENTIATE PRODUCED BY EXTENDING, IN RECURRING MANNER, THE TWO GROUPS OF THREE CIRCLES SHOWN IN THE DETERMINATOR

The pattern elements so produced are indicated by blackening. At lower right is shown an element arising from components of another type.

*Received for publication on September 15, 1940.

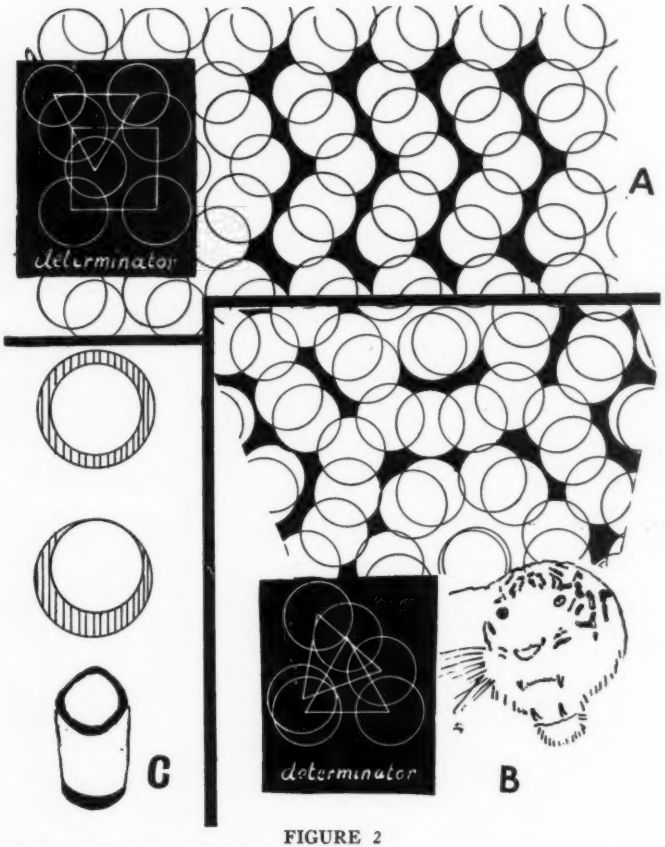

FIGURE 2

A, differentiate containing parallel zigzag formation.
B, differentiate containing spots, and stripes at angles, as upon face of tiger (traced from a photograph).
C, *above*: ocellus from concentric circles; *centre*: excentric ocellus, or can be regarded as crescentic spot; *below*: ocellus, with adjacent crescent, of *Telea polyphemus*, in relation shown above.

within the other; (*b*) arcs of two intersecting circles whose concavities face in the same direction (Figure 1, *a*), forming a crescentic patch (burrowing owl: Figure 4, *f*; *Papilio machaon; Phasianella pullus:* Figure 4, *c*); (*c*) more than two arcs similarly disposed (Figure 1, *b*), forming a winged patch (killer whale, upper patch: Figure 4, *d*; goshawk: Figure 4, *g*); (*d*) two arcs whose concavities face in oppo-

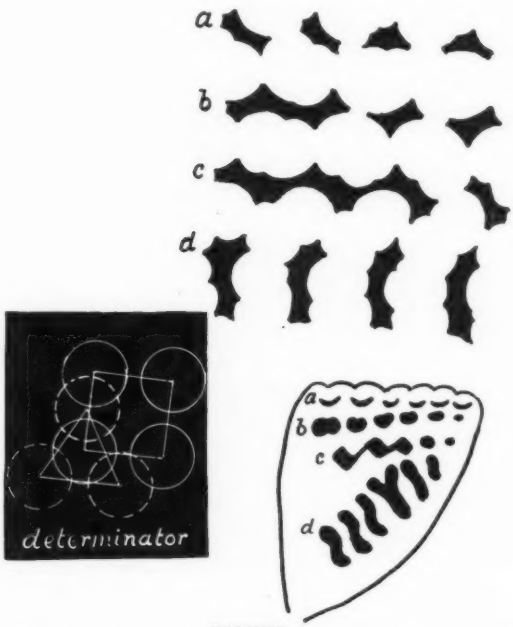

FIGURE 3

Above: differentiate (obtained by tracing) having a resemblance to wing pattern of *Argynnis cybeli; below*: corresponding portions indicated by letters.

site directions (Figure 1, *c*) forming a lenticular, oval or nearly circular marking (loon: Figure 4, *k*), or a patch with two indentations (Figure 1, *f*; killer whale, lower patch: Figure 4, *d*; orchid *Oncidium zebrinum:* Figure 4, *j*); (*e*) a succession of arcs (Figure 1, *g*) forming a crenelated stripe (loon: Figure 4, *k*); (*f*) arcs of circles (necessarily more than two) whose concavities face outwards (Figure 1, *d*) forming a cusped spot (goatsucker: Figure 4, *e*) or cusped stripe (Figure 1, *e*; domestic turkey; face of tiger: Figure 2, *B*; harp seal: Figure 4, *h*; viper; *Helix aspersa*). In such stripes, from their relationship to circular elements, the cusps, or salient portions will reach out as if to join those of the next stripe (turkey; Catocala moth; *Pectunculus glycinus; Dreissena polymorpha; Haliotus tuberculata:* Figure 4, *i*; hooded seal), but the line of junction may lie obliquely to the general direction of the stripe, giving a pattern (Figure 2, *A*) in which projecting angles fall opposite re-entrant angles (*Phasianella pullus,* striped variety). (*g*) Arcs of great diam-

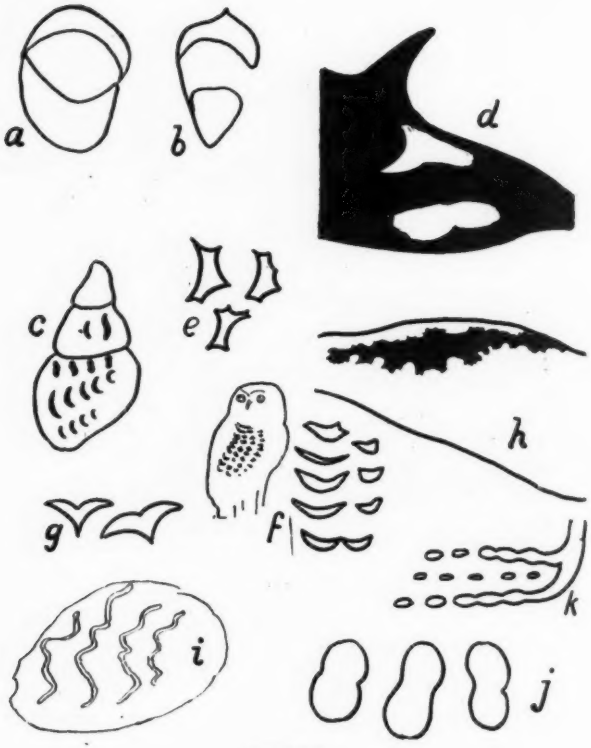

FIGURE 4

a, Wing marking in *Telea polyphemus* suggesting two interacting circles.
b, In *Argynnis cybeli* (hind wing).
c, *Phasianella pullus* showing markings (in order from above) corresponding to elements *g*, *f*, and *a*, of Figure 1.
d, Light areas on side of killer whale.
e, Cusped spots of goatsucker.
f, Burrowing owl (*Speotyto cunicularia*) with crescentic and cusped spots (magnified at right.)
g, Winged spots of goshawk.
h, Cusped and indented patch on harp seal (*Phoca groenlandica*).
i, Zigzag stripes, salient angles opposite, of *Haliotis tuberculata*.
j, Petal markings in *Oncidium zebrinum*.
k, Crenelated striples on loon (*Gavia stellata*) at base of neck.

eter whose centres lie close together, giving narrow, nearly straight stripes (humpback whale, zebra). The zebra occasionally shows a sinus-like indentation in a stripe, further supporting the view that a circular element enters into all types of pattern. (*h*) Arcs in regions

of minimum stagger producing a reticulate pattern (Figure 1, *h*; lizard *Heloderma suspecta;* cone shells).

B. Theory of Differential Periodicity

The object of this paper is to show that the foregoing features can be expressed as the interaction of differential periodic components. As such a demonstration would convey no direct meaning unless the nature of these components could be suggested, it may be explained that the derivation of morphological pattern in this way is postulated in the recently enunciated theory of differential periodicity (Sleggs, 1939).

This theory maintains that the genes lie upon the chromosome, or spireme, at varying angles of rotation stagger, such a conception finding observational support in the various recorded patterns of zigzag and spiral chromosomes (Belling, 1926; Darlington, 1932). The continued production of new gene columns in the process of growth is equivalent to the extension of protein lamellae interacting in rotational stagger.

Since the periodicities of the layers (in respect of the recurring units such as the amino-acid residues) which arise through rotation are in general incommensurable, the gene column exists in a condition of chemical strain which gives it its high synthetic activity. This activity changes in its pattern from cell to cell, and the basis of accurate architecturality from which it proceeds determines a morphogenetic field in the form of a compound differential periodic wave field whose ultimate components represent the interaction pattern between two successive genes.

Now, taking an amino-acid residue, projecting from a protein lattice, if we draw a circle round it, the circumference depicts a contour in the potential degree of stagger which a similar unit of a second lattice may have when the two lattices interact. If circles are drawn round the units of both lattices, the combined system of circles depicts the contours in the morphogenetic field so resulting, the contour changing in value at the point where circles intersect. Hence, by shading areas between the contours, a plotting is obtained of the morphogenetic field in respect of a certain range of synthetic activity.

This has been done, as shown in the figures, schematic components of evenly spaced circles having been used. These do not depict completely the structure of a protein lamella, but we have found that so long as regularly recurring circles are used, irrespective of whether

their arrangement depicts the protein, the pattern produced is, apart from minor details, essentially the same in all cases.

The components to which these living patterns are assigned are, however, not considered to be related to the interaction between two genes only. They are probably components which themselves are compound. The reason for so considering them is related to the production of patterns on various scales and sometimes of great size. Thus the patch on the killer, which measures several feet, is seen to be the equivalent of a mottling upon the goshawk, or *Papilion machaon*.

These varying scales are possible from the fact that the differentiate from two genes has areas of minimum stagger whose geometrical distributing depicts the protein lamella on a scale related to the angle of rotational stagger. These areas can interact with similar areas of another primary differentiate to give a secondary differentiate in which the pattern is further magnified, and it can be shown mathematically that the magnification of any pattern element can proceed, on an infinitesimal scale, to any size whatsoever. If the compound components are of different period, they may produce pattern also without rotational stagger, but the difference has been previously established by rotation in the primary components.

Where the pattern exists in a region of differential growth rates, it will undergo distortion from a simple geometrical pattern (Figure 3). Otherwise the evidence suggests that all patterns can be resolved into rotational components, either two in number, as utilized here for simplicity in a preliminary paper, or of a small number.

C. Summary

Evidence is presented for the generalization that pigment markings have their boundaries formed out of arcs of circles, whose convexity or concavity is directed towards the interior of the patch. These arcs are portions of interacting systems of evenly spaced circles, the systems, or components, being usually present in rotational stagger. Where the pattern is distorted by differential growth rates, the components can nevertheless be identified through the broad correspondence of the pattern with some portion of a theoretical geometrical differentiate derived from such systems of circles.

The rotational components originate in the genar system whose extension in growth is equivalent to the extension of superposed protein lattices in rotational stagger. The fact that the pattern can be depicted by circular elements expresses that a unit, such as an amino

acid residue, of the gene, when interacting with a unit of the adjacent gene, will suffer increase in chemical strain, and hence potential synthetic power, according to the extent to which the two depart from alignment, so that a circle depicts a degree of synthetic activity in the morphogenetic field.

This principle can be extended to the interaction of numbers of genes, permitting of the production of patterns on different scales of magnification.

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Growth, 1941, **5**, 61-67.

RESPONSES OF OVARIAN INTRAOCULAR TRANSPLANTS TO GONADOTROPINS* [1]

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The "law of endocrine deficiency," formulated by Halsted in 1909, is of respectable antiquity among the doctrines of endocrinology. This "law" states that an endocrine deficiency must exist before transplants supplying the deficient principle can be successful. Confirmation of this general thesis has been afforded by Halsted (1909) for the parathyroids; Ingle and Cragg (1939) for the thyroid; Ingle and Higgins (1938) and Turner (1940) for the adrenal cortex.

The present study was undertaken to compare the functional capacity of intraocular ovarian transplants in the presence or absence of a normal ovary in the abdomen.

A. METHOD

From each of 20 virgin female rabbits the left ovary was removed and two pieces of the cortex, approximately 2 mm. long, 1.5 mm. wide, and 1.0 mm. thick, were transplanted autoplastically into the anterior chamber of each eye. Similar pieces were implanted in the spleen, the kidney, and the subcutaneous connective tissue. After a postoperative period which varied from five days to six months, the area of the intraocular transplants was recorded photographically with a Reflex 35 mm. camera (Exakta) and the images measured with the planimeter. Since the initial magnification on the negative was 1.80 times and the photographic negative was projected to a table at a magnification of 10 times, the area was determined and recorded in square inches $\times$ 18.

A control period of daily measurements extending for a week or more was run to determine the stability of the transplants as to size and shape. To minimize areal modifications by atypical vascular changes, no anesthesia was used during photography, the rabbit being placed in a box in which only the head was immobilized.

For purpose of histological study each set of four intraocular trans-

*Received for publication on January 22, 1941.
[1]This study was aided by grants from the Committee on Problems of Sex of the National Research Council.

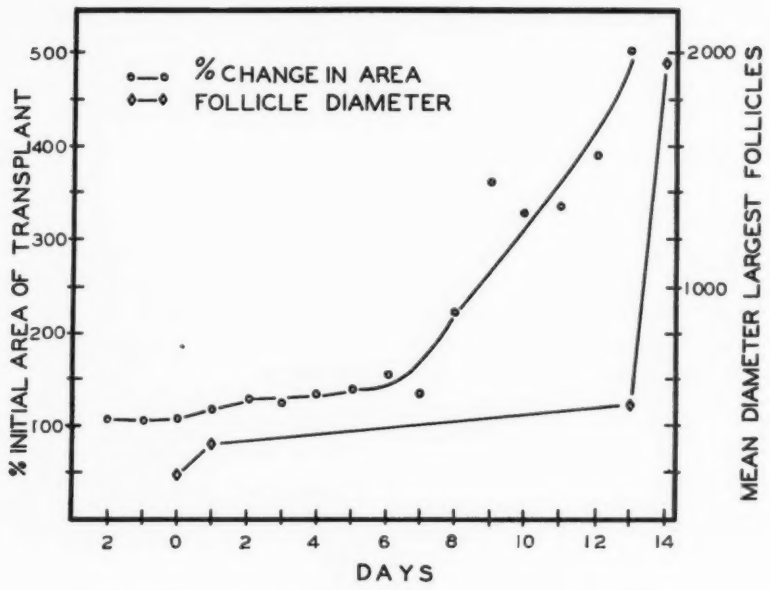

FIGURE 1

RELATIONSHIP BETWEEN THE GROSS CHANGES IN AREA OF THE TRANSPLANTS, AND THE SIZE OF THE LARGEST CONTAINED FOLLICLES

Each plotted point represents an average of all determinations made for a given day. The first injection of Antuitrin *S* occurred on Day 0 and the second injection on Day 13. Follicle diameter is recorded in microns.

plants was removed in the following succession: (*a*) one at the end of the control period; (*b*) a second along with the intact normal ovary and a portion of the uterus 24 hours after the injection of Antuitrin *S* (20 R.U./Kilo) adequate to induce ovulation; (*c*) the third 10 to 14 days later; (*d*) the fourth transplant together with the rest of the uterus was recovered when the animal was killed 24 hours after the injection of a second "ovulating dose" of Antuitrin *S*. At this time the implants to the spleen, kidney, and connective tissue were also recovered. Stained sections of the transplants were studied especially for histological alterations which might be correlated with the recorded changes in area.

B. RESULTS

Nineteen of the 20 rabbits had two or more successful intraocular transplants; 60 of the original 80 were fully established and normal

FIGURE 2

PHOTOMICROGRAPH OF AN INTRAOCULAR OVARIAN TRANSPLANT, SHOWING PARTS OF TWO CORPORA LUTEA, CALL-EXNER BODIES IN THE WALL OF A FOLLICLE, GERMINAL EPITHELIUM, AND A PART OF THE IRIS

FIGURE 3

PHOTOMICROGRAPH SHOWING THE GROWTH CHANGES INDUCED IN A FOLLICLE BY THE ADMINISTRATION OF THE GONADOTROPIC HORMONE WHICH INDUCED THE DEVELOPMENT OF THE ADJACENT CORPUS LUTEUM

histologically. However, in no instance was a transplant recovered from kidney, spleen, or subcutaneous connective tissue found to possess normal ovarian histology, although the sites were positively identified grossly by persisting sutures and microscopically by the fibrotic reaction in the tissues.

Successfully established intraocular transplants have all the elements characteristic of the ovary and exhibit these elements in a pattern indistinguishable from that in the intact gonad (see Figures 2 and 3). Quantitatively the transplants seem to be as well vascularized as the normal ovary. Large vessels may be found which enter the gonad fragment from the iridial plexus and introduce into the substance of the transplant chromatophores which are characteristic of the iris.

The average percentage changes in area of the transplants as well as the average diameter of the largest follicles in the transplants removed at the end of the control period, 1, 13, and 14 days after the first ovulation of the intact ovary are recorded graphically in Figure 1.

A slight increase in the area of the transplants occurs immediately after the first injection of Antuitrin *S*, even when there is a normal ovary present in the abdomen. Following the removal of the abdominal ovary the transplants rapidly increase in size and this increase has continued for the duration of the observations. The average diameter of the largest follicles present in the transplant increases 50 per cent during the 24 hours following the initial injection of Antuitrin *S* and this follicular growth is temporally associated with the initial increase in gross area of the transplant. The rapid increase in size of the transplant after the removal of the normal ovary is not paralleled by a similar increase in the size of the larger follicles of the transplant, since between Days 1 and 13 the average diameter of the largest follicles increases 63.5 per cent and the area of the transplants increases almost 500 per cent.

Table 1 shows the average of the five largest follicles from each of

TABLE 1

AVERAGE DIAMETER IN MICRONS OF THE FIVE LARGEST FOLLICLES IN EACH OF FOUR SERIES OF TRANSPLANTS

Series 1		Series 2		Series 3		Series 4	
Average	Range	Average	Range	Average	Range	Average	Range
190	75-600	293	125-750	461	225-1560	1,937	1,170-2,900

Series 1—Right ovary intact and before the first injection of Antuitrin *S*.
Series 2—Right ovary still intact, 24 hours after injection of Antuitrin *S*.
Series 3—12 days after the removal of the right ovary.
Series 4—24 hours after the second injection of Antuitrin *S*.

four sets of the transplants. The average size of the largest follicles in transplants from animals with a surviving intact normal ovary was only 190 microns, save in one animal in which the diameter of one follicle slightly exceeded 500 microns. When animals possessing such transplants are injected with a dosage of Antuitrin *S* which produces an average of eight ovulation points in the intact ovary, there is never any ovulation in the transplants. Indeed, the average size of the largest follicles in the transplants is increased only 100 microns by this treatment. Following removal of the intact ovary the follicles increase in size approximately 100 per cent. After this period of compensatory hypertrophy,[2] injection of the amount of Antuitrin *S* which had previously produced ovulation in the ovary *in situ* now produced ovulation in the remaining transplant in the two rabbits in which the follicles were at the optimum size and in the other 19 the follicles, which had passed beyond the optimum size for ovulation, became hemorrhagic.

C. Discussion

So long as a normal ovary remains in the abdomen intraocular ovarian transplants in the rabbit would appear to react much like the intact ovaries of hypophysectomized rats. Thus the small average follicular diameter and the atresia of the few follicles that reach 300 microns in the intraocular transplants in the rabbit recalls the data reported by Swezy (1934), Lane and Greep (1935) and others who have shown that the ovary of the hypophysectomized rat is able to maintain and develop quite normal follicles of between 200 and 300 microns diameter, but that follicles larger than this undergo prompt post-operative atresia.

That these small follicles are physiologically still capable of responding to stimulation is indicated by their growth of approximately 100 microns when adequate levels of the urinary gonadotropin Antuitrin *S* is administered, and by their rapid growth response when the intact ovary is removed.

The reasons why the intraocular transplants fail to respond significantly to a dose of Antuitrin *S* which produces an average of eight ovulation points in the intact ovary are at present obscure. One possible explanation is that there are no follicles in the transplant large enough to respond to the injected hormone. The follicular deficiency might be due to an inability of the transplant to respond to hormone levels which produce preovulatory follicles in the intact ovary, or to

[2] A discussion of the factors involved in the hypertrophy of the transplants and follicles after the removal of the surviving ovary will be presented elsewhere.

the utilization of all the autogenous gonadotropins by the intact ovary.

The occurrence of ovulation in only two of the 19 animals, and the marked follicular growth and frequent intrafollicular hemorrhage in the others, is probably due to the degree of follicular differentiation existing at the time of injection of Antuitrin *S*. In an animal, like the rabbit, in which ovulation is not spontaneous, there are three possible responses to the administration of gonadotropic hormone: if the follicles be smaller than the optimum for ovulation, as in an intraocular transplant in an animal with a normal ovary, the follicle may react only slightly; if the follicles are already at the optimum size for the initiation of the ovulatory process, ovulation will be produced; if the follicles are larger than the optimum, they become hemorrhagic.

The fact that the follicles in the transplants in a rabbit with a normal ovary remain small like those found in the ovary of an hypophysectomized rat, and that these follicles or others increase in size and react to gonadotropic hormones after the removal of the remaining ovary would suggest a differential utilization of the intrinsic gonadotropic hormones. The aberrant reactions of the follicles in intraocular transplants cannot be explained by difference in blood supply since the transplant is apparently as well vascularized as the ovary. It seems at present necessary to assume that there is a differential response between the follicles of the ovary and of the transplant, and it is possible that the follicles in the transplant are insensitive to a concentration of gonadotropic hormones which will maintain reactive follicles in the intact ovary.

D. Summary and Conclusions

1. In all of the 60 surviving intraocular ovarian transplants in 20 rabbits, all the elements normally present in the ovary were arranged in characteristic pattern. In none of 20 subcutaneous transplants, 15 intrarenal transplants, and in five intralienal transplants was any normal ovarian histology found.

2. In the presence of a normal intact ovary, intraocular ovarian transplants fail to respond to extrinsic gonadotropic stimulation, either by significant follicular growth or by ovulation.

3. This failure of response is due, at least in part, to the failure of the transplanted ovarian tissue to maintain follicles larger than those characteristic of an hypophysectomized animal. Gonadotropic administration does not induce significant growth changes in follicles of this size.

4. When the only ovarian tissue of the animal is present in the

form of intraocular transplants, these then respond to gonadotropic stimulation in the characteristic way by heightened follicular growth and by ovulation.

5. It is suggested that gonad transplants which are still capable of responding do not do so in the presence of a normal intact ovary because of differential utilization of autogenous gonadotropins.

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Growth, 1941, 5, 69-83.

THE INFLUENCE OF l-PROLINE ON THE GROWTH OF SPONTANEOUS TUMORS IN THE MOUSE*[1]

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A. INTRODUCTION

It is conceivable that if cancer cells could be brought to greater differentiation, cancer growth and spread might be retarded (and life prolonged and made more bearable) since the greater the differentiation the less the potentiality for proliferation.

It would be preferable to use in this connection an agent which nature herself uses for the enabling of differentiation rather than any artificial product, and thus avoid any possible deleterious effects on other growth or physiological processes.

It is from this point of view that we have been systematically exploring the possible rôle in developmental growth of the naturally occurring tissue components of general distribution.

Out of this has come the finding that l-proline—a common amino acid constituent of living tissues—acts to forward structural differentiation of the marine hydroid *Obelia* (Hammett and Collings, 1937).

Further studies by Miller and Reimann (1940) on rabbit ova, by Wilson on Drosophila larvae (1941), and by Hammett, Hammett, and Goldsmith on the hermit crab (1940) have confirmed in principle the findings with *Obelia*. But it was the results with the last named organism which allowed us to institute the trials on mouse tumor growth and these are the subject of the present report. These experiments with lower forms are designed to provide a background from which an understanding of the participants in cancer growth in man may be extended. The focus of attention is on the relation between malignancy and the primary components of growth: those primary components without the full expression of which no production of a complete, ordered, non-pathological organism is possible. A report on the relation between sub-oxidized sulfur groups and cell increase in number in tumor tissue has already been given (Staff of The Lankenau Hospital Research Institute, 1936). The report on the

*Received for publication on January 27, 1941.
[1]Aided by a grant from the International Cancer Research Foundation.

relation between l-proline and cellular and structural differentiation in like material is herewith presented.

B. Material and Methods

The mice used in these trials were the descendents of a single pair known as the *D*-strain. Only spontaneous mammary tumors of females were used. On discovery of a tumor its length, depth, and width were measured with calipers and millimeter scale. These were made each alternate week-day thereafter. The animal was given a special mark and put in a separate cage. Body weight was taken at the beginning and weekly thereafter. The test mice were injected daily with 0.2-0.25 cc. of a freshly prepared M/125 solution of l-proline in distilled water. The injections were subcutaneous between the scapulae. The l-proline was a Hoffman, La Roche product, identified and recrystallized by a member of the chemical staff of the Institute. Diet and environment were the same in general for test and control mice simultaneously. The mice were allowed to live their natural span save a few which were killed when moribund. Age at tumor incidence and death were recorded. The tumors were removed at death, fixed in Susa's, embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

C. Results

In order to save valuable space tabulations will be limited. Statements made in this section have numerical backing in the records. These are on file and may be consulted.

1. *Body Weight*

The mean body weight at beginning and end of experiment was the same for tests and controls.

2. *Tumor Number*

The mice receiving l-proline developed more secondary tumors. This happened not only in this series, but also in a preliminary series run at North Truro for the purpose of establishing the non-toxic dosage. The figures are given in Table 1.

3. *Age Incidence of Tumors*

Test mice did not differ from control with respect to mean age at which the first tumor appeared (Table 2).

TABLE 1

THE NUMBER OF TUMORS PRODUCED BY THE CONTROL AND l-PROLINE MICE

	Total number of mice used	Mice with 1 or more tumors	2 or more tumors	3 or more tumors	4 tumors
			Preliminary Series		
Control	20	20 = 100%	4 = 20%	0 = 0	0 = 0
l-Proline	24	24 = 100%	19 = 79%	6 = 25%	1 = 4%
			Present Series		
Control	76	76 = 100%	34 = 45%	10 = 13%	2 = 3%
l-Proline	78	78 = 100%	44 = 56%	16 = 21%	7 = 9%

TABLE 2

THE AVERAGE AGE IN DAYS AT WHICH THE SUCCESSIVE TUMORS APPEARED IN THE MICE WITH ONLY ONE, TWO, THREE, OR FOUR TUMORS AND THE AVERAGE AGE AT WHICH THE FIRST TUMOR APPEARED IN THE CONTROL AND l-PROLINE MICE

Mice with			Control	l-Proline	Proline minus control
1 tumor	Number of mice		42	34	—8
	Day of appearance of tumor		318	295	—23
2 tumors	Number of mice		24	28	4
	Day of appearance of	1st tumor	310	321	11
		2nd tumor	338	359	21
3 tumors	Number of mice		8	9	1
	Day of appearance of	1st tumor	318	324	6
		2nd tumor	351	353	2
		3rd tumor	365	367	2
4 tumors	Number of mice		2	7	5
	Day of appearance of	1st tumor	308	337	29
		2nd tumor	333	357	24
		3rd tumor	341	365	24
		4th tumor	347	379	32
Average for all first tumors	Number of mice		76	78	2
	Day of appearance		315	312	—3

4. *Survival*

Table 3 gives the average survival in days of the two series of mice after they got their first, second, third, and fourth tumors grouped according to age at first tumor appearance. The figures show a distinct trend to prolongation of life in the l-proline sets.

When the mice are grouped according to number of tumors per mouse the same trend is shown. Thus, of those with only one tumor, the average survival of the controls was 62 days, that of the l-proline 68: of those with only two tumors, the controls lived 62 days, the

TABLE 3

THE SURVIVAL IN DAYS AFTER APPEARANCE OF THE FIRST, SECOND, THIRD, AND FOURTH TUMORS GROUPED ACCORDING TO AGE AT FIRST TUMOR APPEARANCE

Age groups in days	First tumor					Second tumor					Third tumor					Fourth tumor				
	Number of mice		Days of survival			Number of mice		Days of survival			Number of mice		Days of survival			Number of mice		Days of survival		
	Control	Proline	Control	Proline	Proline minus control	Control	Proline	Control	Proline	Proline minus control	Control	Proline	Control	Proline	Proline minus control	Control	Proline	Control	Proline	Proline minus control
170-219	6	10	86	73	*—13*	3	5	66	48	*—18*	1	1	16	49	*33*	0	0	—	—	—
220-269	19	17	52	76	*24*	9	6	30	37	*7*	2	2	25	44	*19*	1	2	32	34	*2*
270-319	15	14	53	65	*12*	5	9	40	41	*1*	0	3	—	24	—	0	1	—	9	—
320-369	16	17	71	73	*2*	7	11	34	34	*0*	4	5	28	39	*11*	0	1	—	44	—
370-419	13	13	76	66	*—10*	9	8	32	41	*9*	3	4	29	36	*7*	1	2	17	9	*—8*
420-469	6	5	54	69	*15*	0	3	—	32	—	0	1	—	28	—	0	1	—	19	—
470-519	1	2	42	59	*17*	1	2	22	40	*18*	0	0	—	—	—	0	0	—	—	—
Total or average	76	78	62	69	*47*	34	44	36	39	*17*	10	16	25	36	*70*	2	7	25	23	*—6*
Weighted mean			63	70	*7*			36	39	*3*			26	35	*9*			25	22	*—3*

l-proline 72: of those with only three tumors, survival was 72 days in the controls, and 78 days in the l-proline: and of those with four tumors the controls lived 64 days and the tests, 64 days. The grand average for the 76 control mice was 63 days, while that for the 78 l-proline mice was 70 days. If the normal life span of a mouse is taken as two years, or 1/30th that of man, the prolongation attributable to l-proline might be considered as approximately seven months of human life.

Two variables may conceivably affect survival, viz., number of tumors per mouse, and age at first tumor incidence. Analysis of the controls gives no evidence the first is significant in this material. It may be that the older the mouse the shorter the survival time. The trend however is uncertain and decision impossible insofar as this material is concerned. If this should be a factor, however, the trend to life prolongation by l-proline is all the more emphasized, since the test mice with only two, three, or four tumors were older than their controls at time of first tumor appearance (Table 2), and hence might have been expected to live a shorter rather than a longer time.

Since, therefore, the basis of the increased survival in the l-proline series cannot be stated to lie either in tumor number per mouse or in age at time of first tumor appearance, the result can justly be attributed to the amino acid.

5. *Tumor Growth in Volume*

For this analysis there were available 145 tumors from the 78 l-proline mice and 122 from the 76 controls. Table 4 presents the frequency of their distribution among the mice of one, two, three, and four tumors according to tumor volume at beginning of record. The average maximum volume is also given for the several groups. These are the most suitable data for comparison of this growth aspect in this material. They show that the tumors of the l-proline series tended to reach a lesser maximum volume. Of the 14 comparable groups where difference obtained, maximum volume was less in the l-proline mice in nine or 64 per cent, and greater in but five or 36 per cent. Comparison of the frequency distribution in tests and controls by the Chi square procedure yielded a P of 0.01, which is statistical verification of the difference (Fisher, 1936).

The average number of days required for the tumors to reach maximum volume is given in Table 5. Apparently this is prolonged somewhat in the l-proline series. Of the 11 comparable groups where difference obtained, the time required in the tests was greater in seven

TABLE 4

DISTRIBUTION OF THE TUMORS AMONG THE CONTROL AND l-PROLINE MICE ACCORDING TO FIRST RECORDED VOLUME AND NUMBER OF TUMORS PER MOUSE, TOGETHER WITH DATA ON THE AVERAGE MAXIMUM VOLUME OF THE TUMORS

Initial volume of tumor	Control: Number of mice with the following number of tumors				Total	Average maximum volume	l-Proline: Number of mice with the following number of tumors				Total	Average maximum volume
	Total 1	2	3	4			Total 1	2	3	4		
						First tumor						
.012-.078	7	5	4	1	17	*7.25*	10	4	3	3	20	*6.71*
.083-.134	12	8	2	1	23	*7.81*	9	8	3	1	21	*5.83*
.146-.175	9	4	1	—	14	*9.76*	8	6	2	1	17	*12.11*
.197-.293	5	3	1	—	9	*8.62*	5	5	1	—	11	*8.82*
.314-.653	7	3	—	—	10	*7.28*	1	4	—	2	7	*10.02*
.791-1.469	2	1	—	—	3	*9.20*	1	1	—	—	2	*8.51*
Totals	42	24	8	2	76		34	28	9	7	78	
						Second tumor						
.025-.078	—	7	1	—	8	*1.82*	—	12	2	2	16	*1.36*
.087-.128	—	6	5	2	13	*1.99*	—	11	6	3	20	*2.21*
.141-.183	—	5	—	—	5	*3.10*	—	2	1	1	4	*2.16*
.197-.263	—	3	2	—	5	*2.08*	—	1	—	1	2	*0.91*
.293-.377	—	1	—	—	1	*3.32*	—	2	—	—	2	*1.82*
.748-1.691	—	2	—	—	2	*3.89*	—	—	—	—	—	——
Totals	—	24	8	2	34		—	28	9	7	44	
						Third tumor						
.016-.065	—	—	1	1	2	*0.10*	—	—	5	4	9	*1.56*
.075-.128	—	—	5	1	6	*0.74*	—	—	3	2	5	*1.77*
.146-.329	—	—	2	—	2	*1.26*	—	—	1	1	2	*0.68*
Totals	—	—	8	2	10		—	—	9	7	16	
						Fourth tumor						
.025-.256	—	—	—	2	2	*3.26*	—	—	—	7	7	*0.48*
Totals	—	—	—	2	2		—	—	—	7	7	

or 64 per cent and less in but four or 36 per cent. Comparison of the frequency distributions by the Chi square procedure gave a P of 0.05, which is statistical verification of the difference. When comparison is made between mice of equal tumor population the same trend appears. Of the 10 pairs the time required for the tests was greater in seven or 70 per cent, and less in three or 30 per cent.

Exact analysis of day by day tumor volume increase, both as individuals and as appropriate averages, failed to disclose evidence that l-proline had consistent influence on rate of tumor size increment. There is therefore no need to give tables or charts of this growth expression.

6. *Tumor Differentiation*

This section is concerned with the possible influence of l-proline on tumor cytology in its relation to differentiation of tumor cells as

TABLE 5

NUMBER OF DAYS REQUIRED TO ATTAIN MAXIMUM TUMOR VOLUME OF FIRST, SECOND, THIRD, AND FOURTH TUMORS ARRANGED ACCORDING TO FIRST RECORDED VOLUME

Vol. Group	1	2	3	4	5	6
			First tumor			
Control	60	63	63	38	41	67
l-Proline	72	61	62	65	55	35
			Second tumor			
Control	42	32	30	22	31	19
l-Proline	34	36	29	29	20	—
			Third tumor			
Control	10	23	10	—	—	—
l-Proline	33	24	18	—	—	—
			Fourth tumor			
Control	22	—	—	—	—	—
l-Proline	20	—	—	—	—	—

expressed by tumor architecture. As everybody familiar with these mouse tumors knows, the outstanding structural characteristic of their developmental progress is the formation of acini. I have therefore taken the extent of acinar development in tests and controls as the index of comparison. Primary is the *extent* to which the acini which are present have developed: secondary is the relative amount of *space* occupied by the acini of any given stage of development in any given section.

It is possible to classify acinar tissue as seen in these tumors under six headings. (*a*) *The pre-acinar.* Here the cells occur in large solid undifferentiated masses. These are easily distinguishable from the common round-cell tumor. (*b*) *The simple clumps.* In these, four to six small round nuclei occur together in separate small irregular cytoplasmic blobs. (*c*) *The compound clumps.* Here from 10 to 20 large nuclei occur in large rounded aggregates with little internuclear cytoplasm apparent. (*d*) *Discrete ring-type acini.* In these there is usually but one row of small cells in cross-section. There may or may not be a central ductulus of lesser or greater diameter depending upon the degree of differentiation. (*e*) *Compact acini.* These are composed of two or more rows of large cells with large nuclei. The acini are closely packed and their ductuli are tiny. (*f*) *Thin ring-shaped acini.* These are probably perversions of the discrete type. The cell band is thin and tenuous, the cells and nuclei are small, and the open space wide and empty.

In this classification there are represented successive steps in acinar development. These are expressions of differentiation activity. It is

possible that two lines of differentiation are exhibited:—one from the pre-acinar mass through the simple clumps to the discrete ring formation, with the thin rings as an off-shoot; the other from the pre-acinar through the compound clumps to the compact. Whether or not this assumption of genesis be correct it is correct to state that the pre-acinar mass is less differentiated than the simple clumps; and that the simple clumps are less differentiated than the discrete and thin ring formations. Similarly the pre-acinar mass is less differentiated than the compound clumps, and the compound clumps are less differentiated than the compact.

The available tumors have been studied in section and placed in the appropriate classes arranged in the stated sequence. The tumors of each class were separable into a dominant and a recessive group. In the dominant group were placed those tumors in which the given stage of acinar development was the major expression of acinar differentiation. In the recessive group of any given class were placed those tumors in which the stage of acinar development represented by the class was second in expression to the major. The figures are in Table 6.

TABLE 6

CLASSIFICATION OF MOUSE TUMORS OF CONTROL AND l-PROLINE TREATED MICE ACCORDING TO DOMINANT AND RECESSIVE CYTOLOGICAL CHARACTERISTICS OF ACINAR DIFFERENTIATION

Stage of Acinar differentiation	Control				l-Proline			
	Dominant		Recessive		Dominant		Recessive	
	No.	%	No.	%	No.	%	No.	%
Pre-Acinar Masses	20	23	2	5	9	10	4	9
Clumped-Simple	22	25	6	15	9	10	11	24
Clumped-Compound	7	8	4	10	15	16	13	29
Discrete Acini	27	31	19	49	37	39	12	27
Compact Acini	9	10	6	15	21	22	3	7
Thin Ring Acini	3	3	2	5	3	3	2	4
Totals	88	100	39	99	94	100	45	100

The table shows that the number and percentage of tumors in which the pre-acinar state was dominant was less in the l-proline than in the control series. This means there were fewer tumors from the l-proline mice in the stage of pre-acinar development. Supportive is the fact that the number and percentage of tumors in which the pre-acinar state was recessive was greater in the test group: for this means that more test tumors were further advanced.

The number and percentage of tumors in which the simple clumped acini were dominant was less in the l-proline than in the control series. This means there were fewer tumors from the l-proline mice in this

early state of acinar differentiation. Supportive is the fact that the number and percentage of tumors in which the simple clump stage was recessive was greater in the test group. This means that more test tumors were further advanced. It is an unqualified result since in no case in this instance was the dominant state composed of pre-acinar masses.

The number and percentage of tumors in which the compound clumped acini were dominant was greater in the l-proline than in the control series. This means there were more tumors in the l-proline mice in this early but more advanced state of differentiation. Since the recessive figures are infiltrated by tumors in which less differentiated states were dominant they are invalid for comparison.

The number and percentage of tumors in which discrete acini were dominant was greater in the l-proline than in the control series. This means there were more tumors in the l-proline mice in this advanced state of acinar differentiation. Supportive is the fact that the number and percentage of tumors in which the discrete acini were recessive was less in the test group. For it means there were fewer test tumors in this class which were dominated by acini of lesser development.

The number and percentage of tumors in which compact acini were dominant was greater in the l-proline than in the control series. This means there were more tumors in the l-proline mice in this essentially complete state of acinar differentiation. Supportive is the fact that the number and percentage of tumors in which compact acini were recessive was less in the test group. For this means there were fewer test tumors in this class which were dominated by acini of lesser development.

There was no difference between test and control mice with respect to the thin ring tumors.

The comparison as a whole gives striking evidence that cytological and structural acinar differentiation has been forwarded by l-proline. This is sustained by Chi square analysis of the frequency distributions. That for the Control-Test dominant groups gives a P of less than 0.01: that for the recessive is less than 0.05. These values establish that distribution of Test data differs from that of Control beyond peradventure.

The presence of a ductulus in a discrete acinus may be taken as a mark of differentiation just as the absence thereof is indicative of incomplete development. Just to check up on the preceding analysis, counts have been made of the acini with and without ductuli in 12 comparable pairs of control and l-proline tumor sections. It is obvious

that such comparison must be made on sections from pairs of tumors the terminal volume of which was of the same order of magnitude; surely from pairs in which the tumor from the l-proline treated mouse was no larger than that from the control; preferably from pairs in which the test tumor volume was somewhat less. This is necessary in order to obviate any possible differences in developmental attainment which might arise from greater size growth. It is also desirable to use pairs of similar initial volume at time of beginning observation, and pairs from mice of like tumor population. In this way genetic or other differences in tumor productivity may be stabilized for.

The figures in Table 7 were obtained from such pairings. They

TABLE 7

THE NUMBER OF DISCRETE ACINI WITH AND WITHOUT DUCTULI IN COMPARABLE TUMORS FROM CONTROL AND l-PROLINE MICE

	Control			l-Proline			
	Acini		%	Acini		%	Proline minus control
	Closed	Ductuli	Ductuli	Closed	Ductuli	Ductuli	
	88	30	*34*	112	51	*46*	*12*
	77	7	*9*	79	40	*51*	*42*
	20	4	*20*	48	17	*35*	*15*
	115	19	*17*	119	34	*29*	*12*
	122	52	*43*	110	69	*63*	*20*
	110	73	*66*	84	37	*44*	*—22*
	129	30	*23*	110	73	*66*	*43*
	105	28	*27*	72	38	*53*	*26*
	56	20	*36*	119	33	*28*	*— 8*
	74	20	*27*	86	39	*45*	*18*
	105	12	*11*	99	51	*52*	*41*
	119	34	*29*	103	48	*47*	*18*
Totals	1120	329	*342*	1141	530	*559*	*217*
Averages	93	27	29	95	44	47	18

show that the tumors from the l-proline mice had a greater number and a higher percentage of acini with ductuli than was the case in the comparable control tumors. This is additional evidence for the conclusion that differentiation expression has been advanced by l-proline.

It is a trite but pertinent biological truism that as differentiation progresses in one and the same structure the intensity of proliferation subsides. Just to be sure the relations set forth in preceding paragraphs are primary and not secondary to an antecedent push in cell increase in number, I have counted the number of cells (nuclei) in transverse sections of 50[2] acini in each slide from which the data of

[2]In one pair but 20 acini were registered in each section.

Table 7 were gotten. The average number of cells per acinus is recorded in Table 8 for these 12 pairs of comparable tumors.

TABLE 8

THE AVERAGE NUMBER OF CELLS (NUCLEI) PER ACINUS CROSS-SECTION IN COMPARABLE PAIRS OF TUMORS FROM CONTROL AND l-PROLINE TREATED MICE

Pair	1	2	3	4	5	6	7	8	9	10	11	12	Mean	*SD*	PE_M
Control	8.6	9.7	9.1	8.9	8.9	9.7	8.2	8.3	7.5	8.0	8.6	9.8	8.8	±0.68	±0.13
l-Proline	7.0	6.4	6.7	7.4	7.4	7.1	7.0	7.1	7.8	7.2	7.0	9.6	7.3	±0.75	±0.15

The figures show the discrete acini in the tumors from the l-proline mice are composed of fewer cells than are those from the untreated controls. Although the actual difference in cell count is small, it is valid, not only because it is present in 10 of the 12 pairs, but also because the difference between the means is more than seven times its *PE* (Pearl, 1940).

It will be noted that one sample in the l-proline series was much higher than the others. Applying Chauvenet's criterion it seems improbable the variate properly belongs to a random sample of the population under consideration. The statistical limits of this should be from 5.76 to 8.84. Such being the case the high value in the l-proline column can be excluded in calculating constants of the seriation (Davenport and Ekas, 1936). As far as difference in mean cell count per acinus of the two series is concerned, however, it need not be omitted, since as shown above, the difference is quite real even when the aberrant value is included. When it and its paired value are removed the difference is only accentuated, and the difference between the means now becomes more than 10 times its *PE*.

It is worth while, however, to remove the high value when computing the Coefficient of Variation. So doing, it is found this factor is 7.44 in the control acini and 4.91 in the test. In other words cell number per acinus is less variable in tumors from l-proline treated mice than in their comparable controls.

These results are consistent with the preceding evidence. First, because cell number is less, which is a mark of decrease in cell proliferation activity; a natural consequence of cell differentiation. And second, because the variability in cell number is less. This is a mark of stabilization; also a natural consequence of differentiation insofar as cell number is concerned.

D. DISCUSSION

The results herewith presented are not striking as far as the abso-

lute magnitude of the differences produced by l-proline are concerned. They are, however, definitely significant when the kind and consistency of change is considered. It is probable that if the mice had been saturated with the amino acid the extent of deviation from control would have been more marked. That such saturation was not attempted was due to the desire to avoid loss in body weight and the complications such would introduce into interpretation. Under circumstances where this complication can be neglected there is no reason why much larger amounts of l-proline may not be used.

The fact that average body weight of test mice was the same as that of controls at beginning and end of experiment removes this potential factor in tumor growth from further consideration.

The fact that average age of the two series at first tumor record was the same indicates general homogeneity in this factor despite age disparities when the mice are grouped according to tumor population.

We are here concerned with whether or not l-proline affects the cellular and structural differentiation of mouse tumors. The evidence presented in this report is both direct and indirect. The direct evidence is derived from study of tumor sections. The indirect comes from records of cell number, tumor number, maximum volume of tumors, and survival after tumor appearance.

Acinar development was used as direct evidence of differentiation expression in the tumors. It was found not only that the tumors from the l-proline treated mice were architecturally more developed, but also that the acinar units thereof were cytologically more mature. It is therefore obvious that l-proline forwarded differentiation here as it has elsewhere.

This is sustained by the following indirect evidence which derives from the secondary reactions, all of which were consistent in direction with the primary affect.

The acini of the l-proline tumors had fewer cells. An advancing differentiation determines a retreating proliferation, which may be expressed, as here, in fewer cells per structural unit.

The number and percentage of secondary tumors, all of which arose after administration of l-proline was under way, was greater in the test than in the control series. This suggests either that more latent tumor cells or nests of tumor cells were brought to proliferation, or that more body cells were changed to tumor cells. Since there is no evidence cell number was increased, but quite the contrary, it follows that the increase in number of secondary tumors may be attributed to an increased specialization of body cells into tumor cells. Since

such specialization is a mark of differentiation activity (Hammett, 1936), the phenomenon is indirect evidence of the differentiation forwarding action of l-proline in this material.

The maximum volume attained by the test tumors was less than that of their controls. Since size increase of tumors is largely factored by cell increase in number, and since cell increase in number is held back by cell increase in differentiation, the fact that the l-proline tumors failed to attain a maximum volume equal to that of the controls is consistent with the finding that differentiation was enhanced.

The time required to attain maximum tumor volume in the l-proline mice was greater than that required in the controls. Since differentiation slows the rate of proliferation, and since the time required to reach maximum volume is factored by the rate of cell increase in number, a prolongation of the time required for this attainment is evidence consistent with the general thesis.

The l-proline mice tended to live longer than the controls after first tumor inception. It is obvious that the greater the differentiation the less the characteristic tumor cell activity for cachexic toxins, and the less the potentiality for proliferative and obstructive metasteses. Life prolongation therefore is a consistent sequel of increased tumor differentiation.

These data then give sure and consistent evidence that l-proline acts to forward differentiation in the spontaneous tumors of the mouse.

They also show that this naturally occurring component of living tissue of general distribution may well be a factor in tumor productivity. It may indeed be that it is a naturally occurring carcinogenic agent when the soil is adequate for this expression.

It may be noted that these results extend the observations on the specific nature of l-proline participation in developmental growth to another species. They thus strengthen the conclusion that it is the carrier of a chemical group essential for expression of differentiation as a basic growth property of living things.

This conclusion, taken in conjunction with the fact that glutamic acid has a like effect on *Obelia* (Hammett, 1936*a*) and is interconvertible with l-proline and gives rise to the same derivatives, raises the suspicion that perhaps distortion of these compounds may be a factor in tumor growth, despite the present contradictory reports which are appearing in the literature. This matter is as yet far from settled. It could be that the faulty differentiation ordinarily exhibited by tumors is in part a consequence of an abnormality in relation of the differentiation enabling chemical groups derivable from these amino acids in the normal configuration.

E. Summary and Conclusion

Subcutaneous intrascapular injections in non-toxic concentrations of the naturally occurring amino acid l-proline were administered to a series of mice from the time of inception of their spontaneous tumors until their natural death. The effect of this compound on tumor production, growth, and cytology has been determined by comparison with a series of simultaneously living controls.

The data show that l-proline conditioned an increase in cellular and architectural differentiation of the tumors; an increase in secondary tumor productivity; a decrease in maximum tumor volume; and a prolongation of the time required to attain this volume. Some prolongation of life was experienced.

The conclusion is that l-proline acts to forward differentiation expression in the spontaneous tumor of the mouse.

The mouse therefore, does not differ from the lower organisms so far studied, inasmuch as the basic growth activity of differentiation is affected alike in all.

F. Acknowledgments

To Edith Hall Williams, Rebecca Comee, Robert Schopbach, and J. McDevitt are due acknowledgments for conduct of the experiment. The tumor sections were prepared by Nevart Gulesian. The statistical analyses were made by Jane Curtis and Bernice Stevens. To all my sincere gratitude.

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THE INFLUENCE OF dl-METHIONINE SULFOXIDE ON THE GROWTH OF SPONTANEOUS TUMORS IN THE MOUSE*[1]

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A. INTRODUCTION

It has been shown that partially oxidized sulfhydryl will retard cell increase in number (Hammett, 1930). It has been shown that sulfoxide will retard mouse tumor growth (Staff of The Lankenau Hospital Research Institute, 1936). This result was obtained with cystine disulfoxide. The compound, however, is unstable and insoluble (Toennies, G., and T. F. Lavine, 1936; Lavine, 1936). If the principle postulated by this and correlated findings is to have practical value in the field of cancer therapy it is necessary that ". . . a sub-oxidized sulfur compound be developed, the radicle of which will protect the effective group from destruction by the metabolic processes of the body before it reaches the growth; or the radicle of which has a special affinity for the cells of malignant growth and thus brings about a concentration of the inhibitive group therein. . . ." (Hammett, 1930). Methionine, like cystine, is a commonly occurring sulfur containing amino acid. It appears to be an integral constituent of living tissue; a possible precursor of cystine in intermediary metabolism; and capable of supporting growth when cystine is absent from the diet. Therefore, because methionine is a naturally occurring, growth supporting, sulfur containing amino acid it was considered that the effect of its sulfoxide on tumor growth was worth a trial. The derivative was prepared (Toennies and Kolb, 1939) and this paper is a report of its influence on the growth of spontaneous tumors in the mouse. The derivative is water soluble and stable in water solution.

B. MATERIAL AND METHODS

Sixty-five female mice were given daily subcutaneous intrascapular injections of 0.094 millimols pure methionine sulfoxide in 0.1 cc. distilled water from the time of first tumor discovery until death. The mice were the descendants of a single pair known as the Bar Harbor "*D*" strain. The data for analysis consist of the age and weight of

*Received for publication on January 27, 1941.
[1]Aided by a grant from the International Cancer Research Foundation.

the mice at first tumor discovery and at death, the number of tumors per mouse, the age at which successive tumors appeared in mice with more than one tumor, and the size of the tumors on successive days from discovery until death. Size was determined by measuring the length, width, and depth of the tumors with calipers and millimeter scale and computing the volume in cubic centimeters according to the formula given in the cystine disulfoxide report (The Staff of The Lankenau Hospital Research Institute, 1936). Measurements were made three times a week every other day. The data so obtained are compared with like material from 76 control mice which did not receive methionine sulfoxide, but which lived, produced their tumors, and died under like conditions of diet and environment. The test mice will hereinafter be referred to as the *MSO* mice.

C. Results

The two sets of mice, controls and tests, were alike in essential particulars at beginning of experiment. Table 1 shows that Mean

TABLE 1

The Mean Beginning, Maximum, and Terminal Body Weight in Grams of the Control and dl-METHIONINE SULFOXIDE Treated Mice

	Beginning	Maximum	End	Beginning	Maximum	End
			All mice combined			
	76 Controls			65 MSO		
	27.8±0.27	34.4±0.36	32.4±0.41	28.4±0.40	37.1±0.52	34.0±0.50
Series	No.	*Mice with one tumor*		No.	*Mice with two tumors*	
Controls	42	27.7	33.9	32.3	24	28.6	35.6	32.8
MSO	35	28.4	36.0	32.7	15	26.5	37.5	34.9
Series	No.	*Mice with three tumors*			No.	*Mice with four tumors*		
Controls	8	26.3	34.2	32.8	2	27.3	31.9	29.1
MSO	10	28.0	38.8	36.2	4	29.6	35.9	35.3

One *MSO* mouse with 5 tumors, 54.3 grams begin, 54.3 max., 40.0 end.

Body Weight was the same; Table 2 shows that the Mean Age at which the first tumor was discovered was the same; and Table 3 shows that the Mean Tumor Volume at time of discovery did not differ to a significant extent. It will therefore be assumed that the mice were essentially homogeneous at the beginning, and that subsequent differences beyond the limit of reasonable variability may be attributed to methionine sulfoxide.

Table 1 shows that the maximum body weight attained by the *MSO* mice was greater than the control and that this tended to be maintained even unto death. This is consistent with the finding that

TABLE 2

THE MEAN AGE IN DAYS OF THE CONTROL AND *MSO* MICE AT DISCOVERY OF THEIR TUMORS

	1st Tumors		2nd Tumors		3rd Tumors		4th Tumors		5th Tumors	
	Cont.	Test	Cont.	Test	Cont.	Test	Cont.	Test	Cont.	Test
Mice with one tumor	318	316	...	...	...	...	...	...	...	...
Mice with two tumors	310	315	338	345	...	...	...	...	...	...
Mice with three tumors	318	353	351	376	365	398	...	...	...	...
Mice with four tumors	308	287	333	298	341	301	347	317	...	...
Mice with five tumors	...	210	...	220	...	251	...	266	...	266
					All tumors combined by succession					
Weighted Means	316	318	341	345	360	363	347	306	...	266

TABLE 3

MEAN MAXIMUM VOLUMES IN CC. AND THE DAYS REQUIRED TO REACH MAXIMUM VOLUME OF THE CONTROL AND dl-METHIONINE SULFOXIDE TREATED MICE

	Maximum Volume		Days required	
	Cont.	Test	Cont.	Test
		All tumors combined		
	5.80	5.85	45.7	46.8
Succession		Mice with one tumor		
1	9.30	9.38	54.8	61.7
Succession		Mice with two tumors		
1	7.11	6.96	55.7	62.9
2	2.34	4.17	30.9	32.1
Succession		Mice with three tumors		
1	6.30	6.90	65.8	64.7
2	2.49	3.47	33.9	39.0
3	0.80	2.30	18.6	21.3
Succession		Mice with four tumors		
1	2.91	3.20	60.0	39.3
2	0.67	3.75	31.0	30.8
3	0.39	0.44	13.5	20.0
4	3.26	1.45	22.0	11.8
Succession		Mice with five tumors		
1	..	12.04	..	60.0
2	..	2.35	..	53.0
3	..	0.90	..	26.0
4	..	0.08	..	11.0
5	..	0.06	..	3.0
		All tumors combined by succession		
All first	8.12	8.10	56.4	61.0
All second	2.28	3.83	31.5	34.8
All third	0.71	1.67	17.6	21.3
All fourth	3.26	1.18	22.0	11.6
All fifth	..	0.06	..	3.0

methionine sulfoxide may replace cystine in the diet of rats and act to forward body weight growth (Bennett, 1939). It indicates that administration of sulfoxide does not necessarily determine a growth retardation. From the fact that sulfoxide may, however, determine a growth retardation as shown by previous experiments (Hammett, 1930; The Staff of The Lankenau Hospital Research Institute, 1936); from the fact that disulfoxide as found in cystine, and sulfhydryl as found in cysteine and other compounds does determine a growth forwarding (Hammett, 1929); from the fact that both methione and methionine may replace cystine in the diet as a growth forwarding agent (Bennett, 1939); from the fact that oxidized sulfur groups may be changed to reduced sulfur groups both by intestinal bacteria and by living tissue; and from the fact that methionine sulfoxide when fed or given subcutaneously may forward growth; it may be inferred that in these and similar results the sulfoxide is not acting as such but as a reduced derivative. In other words the correlated results from several sources suggest that methionine sulfoxide may be reduced to methionine by the processes of living, and that from this or some other sulfur-containing derivative such as cystine or cysteine, may give rise to a growth forwarding rather than a growth retardation. While this interpretation is consistent with the data so far accumulated its verification has yet to be produced by direct experiment. This, however, should not detract from its usefulness as a point of view for testing.

Table 3 shows that the weighted mean amount of tumor tissue produced by the *MSO* mice did not differ significantly from that produced by the controls. In other words, methionine sulfoxide did not affect the gross tumor tissue production one way or the other. Although these values are unavoidably complicated by the presence of unknown amounts of necrotic tissue, it may be assumed that this factor was of essentially equal participation in both sets since the number of tumors involved was not small. The values are based on maximum rather than terminal volumes, since as is well known, tumor size and body weight may decrease in tumor bearing mice towards exitus. Apparently, then, methionine sulfoxide did not act to forward tumor growth as it did body growth. This difference suggests two possibilities; either the compound was destroyed before it reached the tumor tissue, or it reached the tumors and produced a mixed reaction which balanced out in the totals.

Consistent with this absence of group reaction is the fact, also shown in Table 3, that the mean time required to produce this tumor

tissue was the same in the *MSO* as in the control set. In other words methionine sulfoxide neither delayed nor hastened the gross production of tumor mass. As might be expected from these findings there was no numerically significant difference between the two sets with respect to length of life after first tumor discovery. As Table 4

TABLE 4

THE MEAN SURVIVAL IN DAYS OF THE CONTROL AND dl-METHIONINE SULFOXIDE TREATED MICE AFTER DISCOVERY OF FIRST TUMOR

Series	Mice with One tumor Days	Two tumors Days	Three tumors Days	Four tumors Days	Five tumors Days	Weighted Mean Days
Control	61.6	61.8	72.3	64.0	—	62.9
MSO	67.3	68.8	72.6	43.0	72.0	67.0
Difference	+5.7	+7.0	+0.3	—21.0	—	+4.1

shows, the Mean Survival of the *MSO* mice was but four days longer than that of the controls, and this by itself is of no importance when the variability within each set is taken into account.

Finally, it is a fact as shown in Table 5, that the percentage of

TABLE 5

THE NUMBER AND PERCENTAGE OF CONTROL AND *MSO* MICE WITH ONE, TWO, THREE, FOUR, AND FIVE TUMORS

	One tumor		Two tumors		Three tumors		Four tumors		Five tumors	
	No.	%	No.	%	No.	%	No.	%	No.	%
Control	42	55	24	32	8	11	2	3	0	0
MSO	35	54	15	23	10	15	4	6	1	2

mice with multiple tumors was unaffected by the sulfoxide, although it is a fact that there were more multiple tumors in the *MSO* mice constitutionally so disposed than in the controls. But more of this anon.

In summary then, these data show that insofar as group reaction is concerned, methionine sulfoxide had no effect on tumor growth or survival, and that the only valid response was a slight increase in body weight increment. From this it is evident that sulfoxide attached to methionine is, for all practical purposes, no effective retardant of tumor growth.

The story might end here were it not that two questions must be answered. The first is, why was not sulfoxide here an effective retardant as it has been elsewhere? The second is, why did not

sulfoxide attached to methionine produce the same retardation of tumor growth as it did when attached to cystine?

Let us first examine these mouse tumor data to see if they afford a basis for explanation. Now everyone familiar with mouse tumor work knows that the absence of a knock-out blow is no evidence that an effect has not been produced by any given procedure. He also knows that a small effect may illuminate a trend of significance. And he also knows that even a large effect may be masked and lost to sight by variables difficult to control unless the data are segregated in such a way that now one, now the other variable is stabilized for. The chief variables are, of course, age of the mouse at time of tumor inception, tumor size at time of beginning experiment, and number of tumors per mouse. Because of these considerations it is my custom to subject the mouse tumor data to exhaustive analysis. Not to wring like an usurer every drop of blood from the material to support a thesis; but rather like a connoisseur of jewels to examine each facet presented by the cutting and see whether it reflects and adds to the whole, or deflects and destroys consistency of illumination. Justification for such procedure needs no argument among scientists.

The present data have been so examined. They show that neither the age at tumor inception nor the size of tumor at beginning of experiment are useful adjuncts to interpretation in the present instance since test difference from control in these aspects and in their possible influence on subsequent tumor growth was either inconsistent in direction or so tenuous as to be worthless. I did find, however, that when mean tumor volume was plotted for each successive five-day interval from beginning of experiment until death that the course of increase for all first tumors of the *MSO* mice fell below that of their controls, while that of the second and third tumors was above. These relations are shown in Chart 1, Figures 1, 2, and 3. This gave rise to the suspicion that there was a mixed reaction to the sulfoxide insofar as tumor growth was concerned, and that this was in some way tied up with multiple tumor production. Against this idea was the fact that the fourth tumors, instead of being above the controls, as they might be presumed to be if the suspicion were well founded, were below. For a time the idea was discarded. More critical examination of the fourth tumor data brought out two facts: first that they included but two control and five *MSO* tumors, and second that of the *MSO* group there was record for but two tumors after the first five days and of but one tumor after the first 10 days,

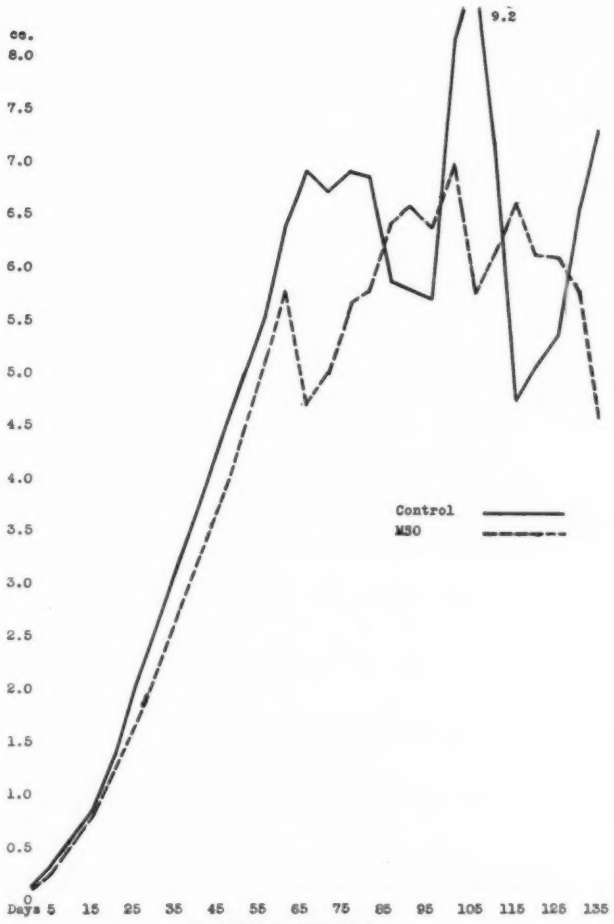

CHART 1, FIGURE 1
THE CHANGE IN VOLUME ON AGE OF ALL FIRST TUMORS
(Values in cc.)

the other mice having died off soon after fourth tumor inception. It is not my custom to draw conclusions from one or two animals. Therefore these fourth tumors will be omitted from this phase of the analysis as being insufficient in number to justify consideration. Their growth, however, is recorded on Chart 1, Figure 4 to make the record complete.

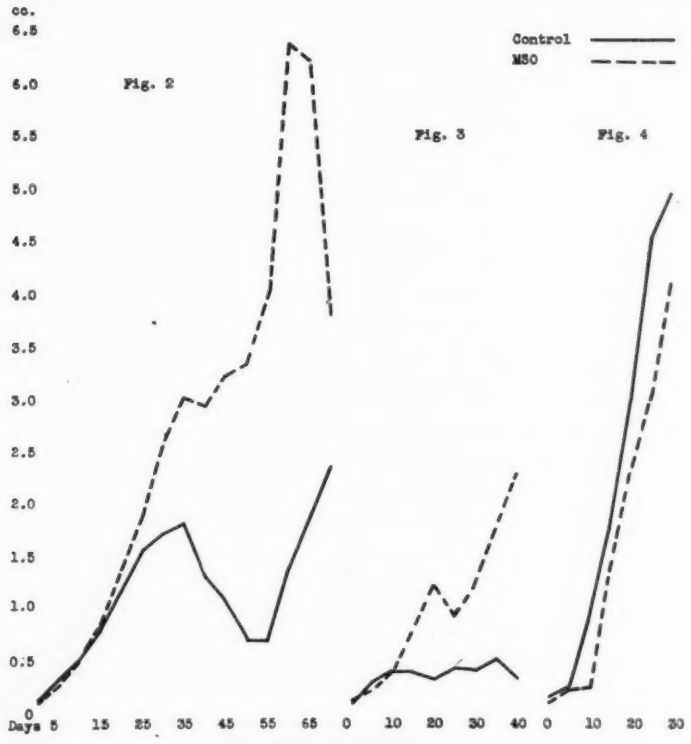

CHART 1, FIGURES 2 - 4

THE CHANGE IN VOLUME ON AGE OF ALL SECOND, THIRD, AND FOURTH TUMORS
(Values in cc.)

Now if methionine sulfoxide produced a mixed reaction, that is to say, a retardation of growth in one group of tumors and a stimulation in another, and if this is related to multiple tumor production, it should follow that either the retardation or the stimulation effect should diminish with increase in tumor number. Whichever happens will depend upon whether multiple tumor production is overcoming retardation or stimulation. Therefore, since the primary property of sulfoxide is retardation rather than stimulation, and since first tumor growth was retarded while that of second and third tumors was stimulated, it would appear as if it were retardation and not stimulation which was being overcome by multiple tumor production. If this

TABLE 6

MEAN SIZE IN CC. OF CONTROL AND *MSO* TUMORS FOR EACH FIVE-DAY INTERVAL FROM DAY OF FIRST MEASUREMENT

Days	Cont.	Test	Diff.	Cont.	Test	Diff.	Cont.	Test	Diff.
	Mice with one tumor			*Mice with two tumors*					
	First Tumor			First Tumor			Second Tumor		
0	0.214	0.176	—0.04	0.185	0.132	—0.05	0.223	0.200	—0.02
5	0.405	0.339	—0.07	0.307	0.268	—0.04	0.440	0.428	—0.01
10	0.706	0.647	—0.06	0.506	0.406	—0.10	0.646	0.805	+0.16
15	1.011	1.020	+0.01	0.817	0.578	—0.24	0.958	1.123	+0.17
20	1.597	1.546	—0.05	1.230	0.865	—0.37	1.356	1.528	+0.17
25	2.556	2.078	—0.48	1.689	1.092	—0.60	1.780	2.226	+0.45
30	3.249	2.804	—0.45	2.348	1.401	—0.95	2.024	3.407	+1.38
35	4.032	3.469	—0.56	2.866	1.967	—0.90	2.134	3.614	+1.48
40	4.474	3.888	—0.59	3.733	2.489	—1.24	1.454	2.821	+1.37
45	4.789	4.600	—0.19	4.835	2.955	—1.88	0.869	4.111	+3.24
50	5.520	5.791	+0.27	5.138	2.819	—2.32	0.879	4.610	+3.73
55	6.277	6.495	+0.22	5.254	3.680	—1.57	0.700	5.356	+4.66
60	7.329	7.150	—0.18	6.142	4.589	—1.55	0.777	6.525	+5.75
65	7.847	4.715	—3.13	6.777	4.822	—1.96	0.977	6.267	+5.29
70	8.190	5.809	—2.38	4.054	3.864	—0.19	1.068	3.812	+2.74
75	8.524	6.384	—2.14	4.540	4.250	—0.29	—	—	—
80	8.110	6.735	—1.38	5.447	4.652	—0.80	—	—	—
85	8.253	7.053	—1.20	5.650	6.215	+0.57	—	—	—
90	8.486	6.901	—1.59	5.648	7.387	+1.74	—	—	—
95	8.524	6.134	—2.39	5.490	8.805	+3.32	—	—	—
100	9.983	5.836	—4.15	2.903	12.098	+9.20	—	—	—
105	10.974	5.800	—5.17	—	—	—	—	—	—
110	8.682	6.172	—2.51	—	—	—	—	—	—
	Consistency of *MSO* deviation from control								
	Plus	Minus	Equal	Plus	Minus	Equal	Plus	Minus	Equal
	2	19	1	4	16	0	13	1	0

TABLE 6 (*continued*)

Days	Cont.	Test	Diff.	Cont.	Test	Diff.	Cont.	Test	Diff.
	Mice with three tumors								
	First tumor			Second tumor			Third tumor		
0	0.103	0.095	—0.01	0.120	0.131	+0.01	0.129	0.150	+0.02
5	0.211	0.186	—0.03	0.260	0.250	—0.01	0.343	0.282	—0.06
10	0.363	0.294	—0.07	0.388	0.399	+0.01	0.470	0.543	+0.07
15	0.610	0.498	—0.11	0.550	0.605	+0.06	0.461	1.065	+0.60
20	0.992	0.747	—0.25	0.940	0.915	—0.03	0.388	1.867	+1.48
25	1.271	1.023	—0.25	1.389	1.575	+0.19	0.616	1.513	+0.90
30	1.154	1.433	+0.28	1.548	2.455	+0.91	0.581	1.740	+1.16
35	1.656	1.985	+0.33	1.594	3.073	+1.48	0.728	2.200	+1.47
40	2.215	2.538	+0.32	1.533	3.486	+1.95	0.599	3.224	+2.63
45	2.939	3.449	+0.51	1.794	3.159	+1.37	—	—	—
50	3.574	3.167	—0.41	0.753	2.390	+1.64	—	—	—
55	3.928	3.638	—0.29	1.148	3.051	+1.90	—	—	—
60	4.366	4.309	—0.06	2.089	6.044	+3.96	—	—	—
65	4.981	4.269	—0.71	—	—	—	—	—	—
70	6.016	4.146	—1.87	—	—	—	—	—	—
75	5.977	5.130	—0.85	—	—	—	—	—	—
80	6.362	4.671	—1.69	—	—	—	—	—	—
85	1.775	4.155	+2.38	—	—	—	—	—	—
90	2.150	4.609	+2.46	—	—	—	—	—	—
95	2.088	3.343	+1.26	—	—	—	—	—	—
	Consistency of *MSO* deviation from control								
	Plus	Minus	Equal	Plus	Minus	Equal	Plus	Minus	Equal
	7	12	0	10	2	0	7	1	0

TABLE 6 (*continued*)

MEAN SIZE IN CC. OF CONTROL AND *MSO* TUMORS FOR EACH FIVE-DAY INTERVAL FROM DAY OF FIRST MEASUREMENT

Days	Cont.	Test	Diff.	Cont.	Test	Diff.	Cont.	Test	Diff.	Cont.	Test	Diff.
						Mice with four tumors						
	First tumor			Second tumor			Third tumor			Fourth tumor		
0	0.060	0.105	+0.05	0.095	0.179	+0.08	0.095	0.102	+0.01	0.201	0.120	—0.08
5	0.083	0.285	+0.20	0.190	0.229	+0.04	0.203	0.167	—0.04	0.290	0.289	—0.00
10	0.096	0.458	+0.36	0.204	0.355	+0.15	0.322	0.194	—0.13	0.820	0.500	—0.32
15	0.140	0.622	+0.48	0.292	0.847	+0.56	0.334	0.162	—0.17	1.789	1.384	—0.41
20	0.212	1.011	+0.80	0.505	1.230	+0.73	0.382	0.250	—0.13	3.002	2.333	—0.67
25	0.227	1.922	+1.70	0.131	2.406	+2.28	0.078	0.223	+0.15	4.559	3.071	—1.49
30	0.245	2.044	+1.80	0.201	0.990	+0.79	0.078	0.455	+0.38	4.910	4.139	—0.77
35	0.395	2.939	+2.54	0.377	1.362	+0.99	0.078	0.524	+0.45	—	—	—
40	0.612	4.672	+4.06	0.460	1.960	+1.50	0.031	0.481	+0.45	—	—	—
45	0.832	0.339	—0.49	0.395	2.573	+2.18	—	—	—	—	—	—
50	1.679	0.301	—1.38	0.403	2.771	+2.37	—	—	—	—	—	—
55	2.685	0.379	—2.31	0.339	4.287	+3.95	—	—	—	—	—	—
60	2.981	0.414	—2.57	—	—	—	—	—	—	—	—	—
65	3.306	0.466	—2.84	—	—	—	—	—	—	—	—	—
						Consistency of *MSO* deviation from control						
	Plus	Minus	Equal	Plus	Minus	Equal	Plus	Minus	Equal	Plus	Minus	Equal
	8	5	0	11	0	0	4	4	0	0	5	1

assumption is correct it should be expected that the retardation of first tumor growth in the *MSO* mice would be greatest in the mice with only one tumor, and progressively less in mice with two, three, and four tumors respectively.

In order to find out if fact meets expectation it is necessary to break down the data from which Figure 1 of Chart 1 was composed into its components. That is to say, to segregate the successive first tumor volumes of mice with but one tumor, from those of the mice with only two tumors, and so on seriatim. This has been done. The results are given in Table 6. Here may be found the direction and mean amount of volume difference of the *MSO* tumors from their controls of the first tumors only of mice with but one, but two, but three, and but four tumors respectively. It may have been noted that there was one mouse in the *MSO* series with five tumors. This mouse was not included in this comparison for the very obvious reason that no comparison could be made since there was no control mouse with five tumors.

Before taking up the results of this segregation I would like to point out that consistency of direction of test deviation from control is as valid an index of difference as is the amount of deviation, and that evaluation of growth reaction to any given procedure must depend on this quite as much as on that, since consistent change in direction of deviation may be expression of introduction of additional factors. The possibility of fallacious interpretation of trends arising from the use of totals of averages of successive increments over an entire growth course should be apparent to all and need no further exposition.

The first tumor data of Table 6 reveal two facts. One is that first tumors in mice with only one, two, or three tumors were retarded by methionine sulfoxide. This is shown by the fact that in these groups test volume was less than control with reasonable consistency. Now there were but two control mice with four tumors, and four *MSO* mice. This small number, combined with the natural variability, makes decision regarding continuance of the trend into this group more suggestive than conclusive. In the table the means for all tumors of this group are given to make the record complete. Taking them as they stand they indicate that *MSO* forwarded rather than retarded these first tumors of the mice with four tumors. But examination of the basic data reveals that the *MSO* values are heavily weighted by one tumor of excessive size as compared with the other three. These lie closely together on all aspects and are comparable

with the two controls. It seems legitimate therefore to delete this extra-large first tumor from participation in the averages. When this is done the marks of growth enhancement disappear, and in their place comes evidence that if *MSO* did not retard growth it did at least not act as a stimulus. Thus there was a total minus differential in volume of the *MSO* tumors of —8.30 cc., and this minus trend appeared in seven or 54 per cent of the 13 opportunities therefor during the course of tumor growth. Furthermore, despite the fact that the mean initial volume of the three *MSO* tumors was twice that of the two controls (viz., 0.11 versus 0.06 cc.), the mean maximum volume reached was but 0.89 cc. or 31 per cent of that reached by the controls. These facts, taken in conjunction with those of the other first tumors justify the assumption that *MSO* may retard first tumor growth in mice with but four tumors as it does with that of mice with but one, two, or three tumors. The results afford pretty consistent evidence that *MSO* acts to retard growth of first tumors in general.

Now there are certain data consistent and therefore sustaining of this conclusion. These are the data relating to maximum tumor volume, survival, and tumor cytology of mice with only one tumor. Obviously like data from mice with multiple tumors cannot be used in this connection since, as will appear, multiple tumors have an influence on first tumor reaction.

Table 3 shows that the time required by the *MSO* tumors to attain maximum volume was lengthened over the controls. This is evidence consistent with the idea of growth retardation. Now it could be expected that retardation of tumor growth might act to prolong life. The *MSO* mice did live on the average almost six days longer than their controls (Table 4). While this fact alone is of no importance, it is by its presence another straw indicating the wind was blowing towards retardation.

Since sulfoxide has been shown to retard cell multiplication, examination of tumor sections for evidence of like action here was indicated. The available material was scanty. Nevertheless six pairs of tumors of comparable size and architecture were found and counts were made of the number of acini in 10 fields of each, and of the number of cells per acinus in 100 acini in each. The technic was the same as that used in the cystine disulfoxide study (The Staff of The Lankenau Hospital Research Institute, 1936). In these six pairs the average number of acini per field was 21.5 for the *MSO* and 26.5 for the control mice: and the average number of cells per acinus

from the *MSO* tumors was 6.8 while that from the controls was 8.4. These figures, though derived from all too little material, are at least consistent with the evidence of first tumor growth retardation. Finally, the retardation was effected without evidence that the body weight of the *MSO* mice was adversely affected (Table 1). There is therefore no evidence inconsistent with the conclusion that methionine sulfoxide induced retardation of first tumor growth in these experiments.

The second fact shown by Table 6 is that the first tumor retardation was greatest in mice with only one tumor, and progressively less in those with two, three, and four tumors respectively. Consider the consistency of deviation. In the mice with but one tumor the deviations were towards a lesser volume in 91 per cent of the cases: in those with two tumors this dropped to 80 per cent; in the three-tumor mice this receded to 63 per cent; and using the adjusted values for the four tumor mice it fell to 54 per cent.

Therefore, the fact that first tumor growth was retarded by *MSO*, combined with the fact that the efficacy of the retardation decreased progressively in mice with but two, three, and four tumors raises the suspicion that retardation is overcome by multiple tumor production to the status of a definite possibility.

So far I have not indulged in speculative interpretation but have confined myself to interpretation based on facts provided by the data. Continuing thusly we can see what further examination of the results affords.

Since multiple tumor production is a factor in destroying the retardative influence of methionine sulfoxide it is possible that this effect can be attributed to the mass of tumor tissue produced. If this assumption is correct it should be expected that as the mass of *MSO* tumor tissue increases the retardation of first tumor growth should concurrently decrease.

In order to determine whether this assumption is sustained by the results I have calculated the mean volume increase for each of the successive five-day intervals from beginning of experiment until proliferation is no longer a major growth factor. To those familiar with mouse tumor growth it is common knowledge that proliferation is a major growth activity contributing to size increase until the time when these structures become necrotic as they frequently do, and break down into discharging lumps. Obviously when this occurs the size changes are not very reliable measures of proliferation growth. One index of necrosis is volume decrease. I have there-

fore omitted from the averages all volume decrements over 0.1 cc., and those which occurred at time of death, since our interest is primarily in proliferation expression and not necrotic accumulations. The values used, then, represent as closely as possible the five-day increments in volume of not obviously unhealthy tumors. The values so obtained were charted and examined as to the trend exhibited thereby. With this determined, smooth curves were fitted thereto by the use of appropriate formulae. These curves are given in the accompanying charts together with the equations from which they are derived. There was of course considerable variability but the curves are accurate representations of the trends.

Now if the assumption stated above is correct it should be found that the retardation of *MSO* tumor volume increase would diminish in relation to the controls when the mass of proliferating tumor tissue reaches a certain size. Realization of the expectation in the tumors

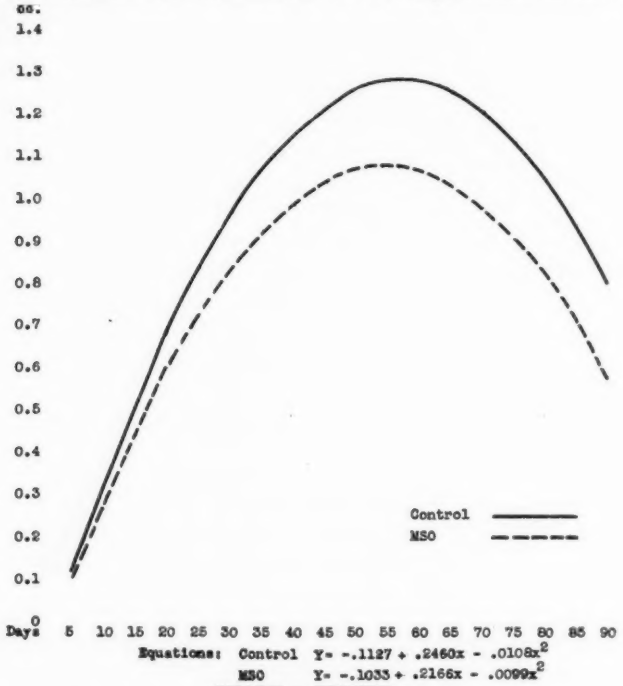

CHART 2, FIGURE 1

FITTED CURVES OF DAILY INCREASE IN VOLUME OF FIRST TUMORS OF MICE WITH BUT ONE TUMOR

of mice with but one tumor would depend only on the mass attainment of the single tumor. Inspection of Chart 2, Figure 1, shows that the expectation was realized. The curves show that whereas the course of volume increment of the *MSO* tumors was less than control throughout, this tended to approach that of the controls in the later periods. Until recession begins it is evident that volume increment of the *MSO* tumors tended to become more and more less than that of the controls, and when it begins it tended to become less and less smaller. This approach can only mean that the retarding influence of sulfoxide diminished when tumor mass reached a certain size.

Now if the assumption that tumor mass acts to overcome the retardative effect of *MSO* is correct, it would be expected that the greater tumor mass present in mice with two and three tumors would produce a greater effect than was found in the mice with but one tumor. Turning to Chart 2, Figures 2 and 3, it is seen that this

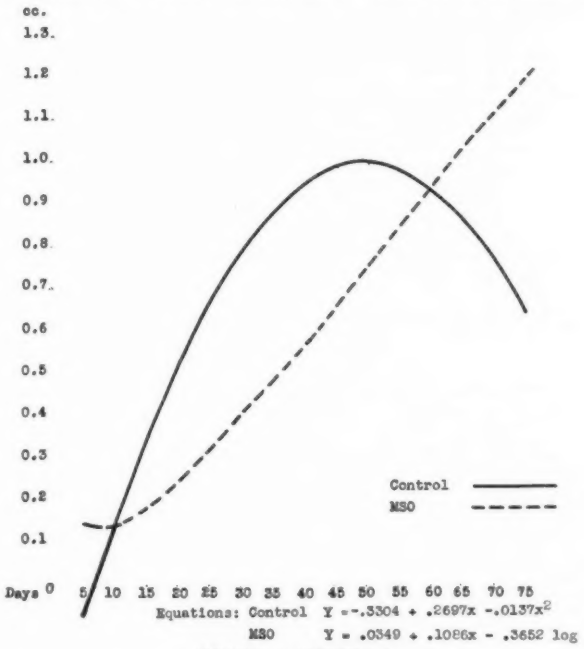

CHART 2, FIGURE 2

FITTED CURVES OF DAILY INCREASE IN VOLUME OF FIRST TUMORS OF MICE WITH BUT TWO TUMORS

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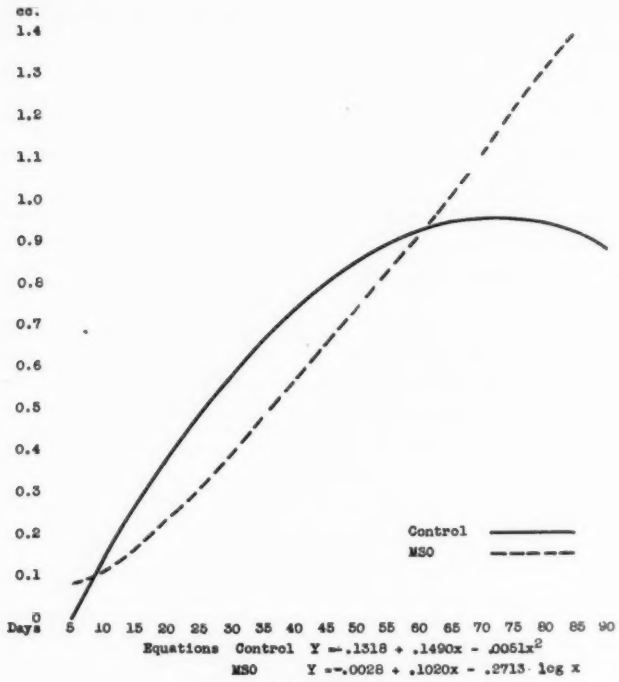

CHART 2, FIGURE 3

FITTED CURVES OF DAILY INCREASE IN VOLUME OF FIRST TUMORS OF MICE WITH BUT THREE TUMORS

expectation is realized. In the first place the *MSO* tumor increments—which started out as they should by being less than the controls, since there was retardation of first tumor growth, approach those of the controls as growth progresses, which is evidence of an overcoming of retardation. But what is more, the increments do not stop at merely approaching those of the controls as they did in the case of the tumors of mice with but one tumor; instead they go on to meet and even pass those of the controls. This is pretty good evidence that the braking influence of tumor mass on the retardative influence of *MSO* is greater in mice with multiple tumors than in those with only one. Such being the case the assumption stated above that tumor mass is a large factor in overcoming the retardative influence of *MSO* is seen to be well taken, and raises what was previously a possibility to the present status of a probability.

The separate extension of the analysis to mice with four tumors was not made because the number of individuals concerned was too few for reliability. But as a final confirmation, curves were made from averages of successive increments of *all* first tumors combined. These are given in Chart 2, Figure 4. They have essentially the

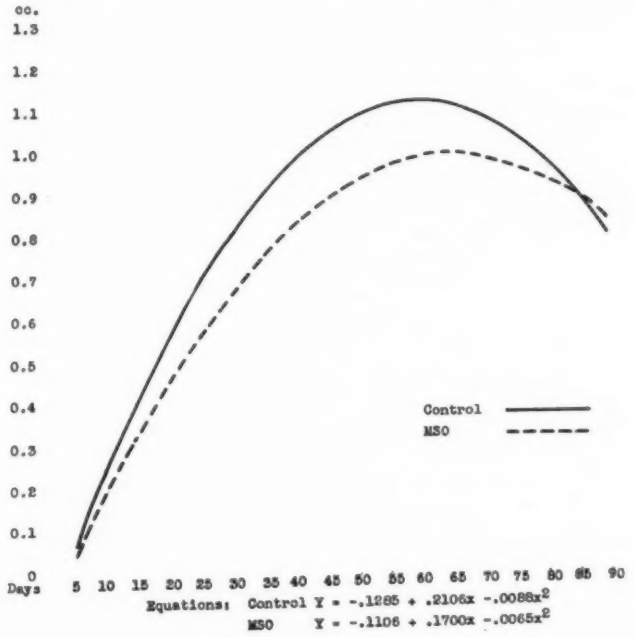

CHART 2, FIGURE 4
FITTED CURVES OF DAILY INCREASE IN VOLUME OF ALL FIRST TUMORS

same form as those of Chart 2, Figure 1, and thus are mutually sustaining. It is not possible to get more out of data by mathematical analysis than is contained in them. Comparison of Figures 1 and 4 show that the *MSO* increments approach more closely to the controls in the later stages when *all* first tumors are combined, than when only the tumors of mice with but one tumor are compared. This is of course as it should be because of inclusion of the greater overcoming effect of the mass provided by the multiple tumors. That the *MSO* curves in Figure 4 follow those in Figure 1 rather than those of Figures 2 and 3 comes from the fact that the values are weighted

means in which the proportionate representation of each group is determined by the number of members thereof.

Now the data of Table 6 and Figures 2 and 3 of Chart 1 show that the second and third tumors of the *MSO* mice grew better than those of their controls, and that consistently. This stimulation reaction, like that of retardation exhibited by the first tumors must have stemmed from the same administered substance since no other deviation from control procedure was exercised. Therefore, since the extent of the retardation response to the administered substance was conditioned by tumor mass production, it should naturally follow that the extent of stimulation reaction would also be affected. And since this influence acted to overcome retardative effectiveness in the slower growing tumors, it could be expected to act on the other hand as a forwarding factor to the faster growing. As Brer Rabbit says: "*So say: so do.*" For when the successive five-day increments of all

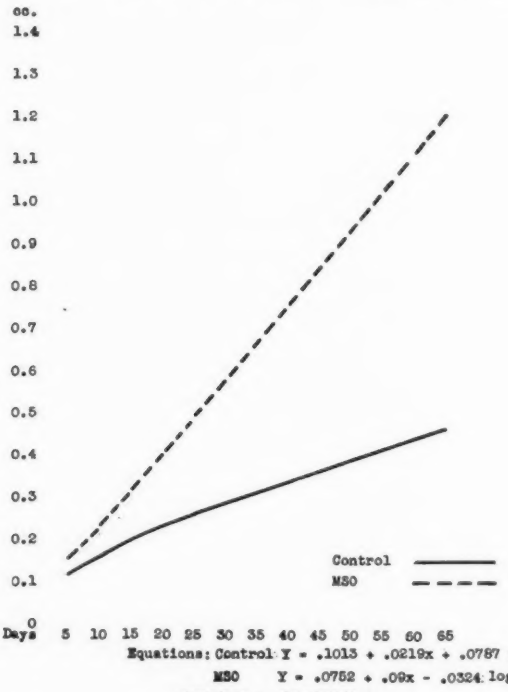

CHART 3, FIGURE 1

FITTED CURVES OF DAILY INCREASE IN VOLUME OF ALL SECOND TUMORS

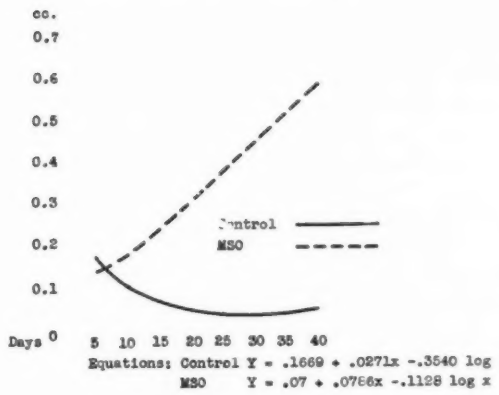

CHART 3, FIGURE 2
FITTED CURVES OF DAILY INCREASE IN VOLUME OF ALL THIRD TUMORS

second and third tumors respectively are plotted and smooth curves fitted to the trend as they have been in Chart 3, Figures 1 and 2, it is found that not only are the *MSO* values greater than the control, as they should be, but that the deviation from control increases as tumor mass increases. Since the *MSO* increments fit an ascending logarithmic curve it is clear that here there has been growth acceleration as well as stimulation. This, in the second tumors, is in contrast to the controls where the increments fit a parabola, which as is well known shows deceleration in the later phases. Deceleration is also evident in the third tumor controls and that from the beginning. Consistent with this idea of acceleration derived from increasing tumor mass is the fact shown in Chart 2, Figures 2 and 3, that first tumor increments in the presence of two and three tumors also draw a logarithmic curve, in contrast to the parabola of the controls.

So then we have the first approximation to an answer to our first question. Methionine is not effective as a growth retardant because the to-be-expected proliferation inhibition of the sulfoxide group is overcome by some factor associated with tumor-mass production. It is this factor which contributes to the observed mixed reaction, i.e., retardation of first tumors and acceleration of second and third; and because of the balancing out of these opposite reactions the end result in the *MSO* mice is no different from in the controls insofar as tumor-mass production is concerned.

So far I have dealt solely with facts and factual interpretation,

and facts presented by the results of this experiment. Other facts from other fields can now be used to give an explanation as to why the retardative effect of sulfoxide in methionine combination is overcome and reversed by increasing tumor mass.

D. Discussion

It is possible and appropriate to explain the results of this experiment on the basis of the relation of the sulfur groups to cell increase in number. Something over 10 years ago I showed that reduced or sulfhydryl (SH) sulfur was a naturally occurring chemical stimulus essential for cell proliferation (1930). Defense of that postulate is no longer necessary since confirmation has come from many sources. I then showed that the partially oxidized products of this group should and would act as retardants of proliferation (Hammett, 1930). This too has been confirmed from other sources, but less extensively. From these findings I concluded that these groups play a significant part in the regulation of cell increase in number, and gave further experimental evidence for the belief. Now the regulatory influence of these groups on proliferation is of course founded in the well established principles of chemical equilibrium. In a concise presentation of the possibilities of change which these groups may undergo in living substance during the passage from the reduced or SH state, to the completely oxidized or SO_4 condition, I emphasized that the intermediate or partially oxidized states, such as are represented by the sulfoxides, the sulfones, and the sulfinates may be subject both to reduction and to oxidation, and that although the ultimate outcome is oxidation, the immediate fate depends on the redox state of the cells in which the groups are present (Hammett, 1930, 1934). On the basis of the then known data I tentatively indicated the predominant trend of each group according to its state of oxidation and the relative potentiality thereof for reduction. The principles therein set forth are as valid and applicable today as they were 10 years ago.

Experiments done at The Marine Experimental Station of The Lankenau Hospital Research Institute, as well as the present results, have justified the prediction then made that the intermediate oxidation products of SH may either be reduced by living substance to SH and act as proliferation stimuli, or may act as such and retard this growth activity before going on to further oxidation and proliferation ineffectiveness. This direct evidence is given by the studies of Toman with regenerating tadpole tails (1939), of Wilson with Drosophila larvae (1933), of Walp with non-nucleated blue-green

algae (1940), and the present experiments with mouse tumors. These results with the latter show that there was effective action, and that the absence of test difference from control in the totals was not due to destruction of effective groups but to a balancing out of opposite responses.

From the studies at The Marine Experimental Station it came out that pH is an important factor in determining which way the cat will jump. Thus we get sustaining evidence for interpretation since Clark (1928) has shown the close dependency of the redox equilibrium on pH. Correlated evidence in the same direction is given by the studies of Voegtlin, Maver, and Johnson (1933), and Chalkley and Voegtlin (1940). These show that the proliferation stimulating effect of SH on nuclear division in amoebae is not only dependent but may even be reversed by shift in oxygen tension.

Having laid the foundation we can now go on to postulate why the to-be-expected retardation effect of methionine sulfoxide was overcome by increasing tumor mass. Otto Folin (1905) in his pioneering studies established a distinction between exogenous and endogenous metabolism for sulfur as for nitrogen. The exogenous processes, concerned with disposal of ingesta, yield end-products which are largely sulfates. Their trend is overwhelmingly towards oxidation. The endogenous processes, on the other hand, have to do with inter- and intra-cellular activities of living, and since these yield incompletely oxidized or "neutral" sulfur in no inconsiderable part, it may be assumed that intermediate reactions are here important. It is with these latter that we are concerned, rather than with the trend as a whole.

From the principles of redox equilibrium set forth by Clark (1928) we know that oxidation and reduction are continuously taking place in the living cell and that the balance may shift now to one side, now to the other. In terms of the sulfur groups and their relation to proliferation this means that when the shift is towards the reducing side conditions are favorable for SH production or maintenance, and proliferation stimulation, while when the shift is towards oxidation, conditions are favorable for SO (sic) production and maintenance, and consequent retardation.

From the fact observed in other materials that SH is concentrated in regions of proliferation activity (Hammett, 1929; Hammett and Chapman, 1938; Chapman, 1937) it may be assumed that a like condition holds in these mouse tumors where proliferation is a major growth activity. Therefore if the proliferation retarding effect of

SH is to be expressed it must reach the tumor in sufficient amount to neutralize the SH effect, or if it be preferred, to change the redox state from its naturally high trend to reduction, to a lesser or even the opposite or oxidation activity. The latter has yet to be attained. For if it were, the tumors of mice receiving SO would grow little if at all. The fact that the first tumors of mice receiving *MSO* continued to grow, though at a lesser rate than the controls, may be taken to mean that the SH effect was only partly neutralized; that the redox state was shifted towards but not to a state of oxidation dominance.

Granting this, a matter of first importance for interpretation is the ratio of SH to SO in regions of tumor cell proliferation. No analytical data are available, nor can there be until appropriate methods are devised. All that can be done therefore is to reason from effect to cause. It has been shown, however, that SH increases with increase in proliferation intensity (Hammett, 1929; Hammett and Chapman, 1938, and Chapman, 1937). It can therefore be assumed that the amount of SH in the nests of cells comprising the young tumors is high, that the absolute amount is greater the greater the mass of proliferating tumor tissue, and that the absolute amount is greater the greater the number of tumors. Concisely, it can be assumed that the absolute amount of SH in the tumors is a dominant variable which tends to increase with increase in size and number of tumors. The amount of SO which was offered to the proliferating tumor tissue in these experiments was, however, essentially a constant. The amount administered to each mouse each day was a constant, and it can be inferred that the amount reaching the tumors averaged up to an essentially constant quantity for any given series of mice. From this it can be assumed that the amount of sulfoxide presented to the tumors decreased relative to the amount of SH contained as tumor number and size increased. It could, therefore, happen, if the SO happened to be particularly reducible because of inadequate protection afforded by the radicle to which it was attached, or if insufficient SO was administered, that the retardative effect would diminish with increase in tumor number and tumor size. And this latter is exactly what did happen when methionine sulfoxide was administered.

It has been found that sulfoxide attached to cystine breaks down in water solution to yield cystine and sulfinate (Lavine, 1936). It has also been noted that partially oxidized sulfur groups may be reduced *in vitro* in the presence of SH. This potentiality for reduction is probably present *in vivo* as *in vitro*. Therefore it can be assumed,

though it is not yet proven, that sulfoxide presented with an overwhelming amount of SH such as could occur in relatively large tumor masses, would itself be reduced. Granting this, it could happen that sulfur originally administered as sulfoxide could be changed to SH (sic) and act as an additional stimulus to proliferation expression. If such should happen it could also happen that there would result acceleration of tumor growth. And this latter is exactly what did happen.

The question might reasonably be asked: How is it that acceleration could be produced when already there is assumed to be present in the growing tumor mass a considerable amount of the stimulating SH? The basic reason which can be given in reply is that common experience shows that under usual conditions the growth potentiality of any organism or part thereof is never expressed to its utmost, and therefore that the addition of a growth stimulus can effect an acceleration, not only with respect to the previous rate of increment of the experimental animal, but also with respect to the control which has not received the added fillip. Subsidiary to this is the fact sketched in preceding paragraphs that the nature of the response is related to the relative amounts of stimulating and retarding groups. And finally the experimental fact that there was acceleration.

While it might have been possible to predict that what did happen to these mouse tumors would happen from the available knowledge of sulfur groups *in vitro* reactions, this was not previously done on paper, and hence the present fitting of theory to result is obviously more a *post hoc ergo propter hoc* explanation than a specimen of inductive reasoning. On the other hand facts do fit theory in this particular instance and while there are many lacunae which remain to be filled by appropriate experimentation, the picture as a whole adds foundation for further exploration.

The question has been asked: Why did sulfoxide attached to methionine not retard tumor growth as effectively as when it was attached to cystine?

It might at first thought be believed that the lesser effectiveness arose from the fact that the "dl" instead of the natural isomer was used. If this were the case no action at all, or only a weak reaction should be expected, but as the results show, there was definite though mixed reaction. The same objection would apply to interpretation of the difference on the basis of the greater *in vitro* stability of the methionine derivative.

Now the results and the analysis thereof given in preceding para-

graphs give pretty definite evidence that not only is the retarding influence of sulfoxide overcome, but that the group is reduced and in the reduced state acts as a stimulus. Since no evidence of such reversal was given in the cystine disulfoxide experiments, the assumption can be made that sulfoxide in methionine is more reducible than sulfoxide in cystine. This is not to say that sulfoxide in cystine disulfoxide is not reducible, it is to say that reduction of sulfoxide in the methionine derivative is reducible to greater effective stimulation concentration. Evidence that sulfoxide in cystine may also be reduced is present in the fact that the inhibitive effect of this derivative was less on second than on first tumors (The Staff of The Lankenau Hospital Research Institute, 1936). But in no case was reduction sufficient to provide stimulation expression.

There is further evidence consistent with the idea that sulfoxide in methionine is more easily or more effectively reducible than sulfoxide in cystine. For Bennett (1939, 1937, 1939a) has found that whereas both methionine sulfoxide and cystine disulfoxide can replace methionine and cystine respectively in the diet of rats, the former is available molecule for molecule, while the latter is only available in the ratio of three for one. That is to say, since the sulfoxide can presumably replace the sulfides as dietary growth essentials only by first being reduced thereto, possibly by the intestinal flora or the liver or both, and since it takes three molecules of cystine disulfoxide to replace one of cystine, while it takes but one molecule of methionine sulfoxide to replace one of methionine, the implication is that the latter is more effectively reduced than the former.

It may therefore be assumed until evidence to the contrary is available, that sulfoxide in methionine is less effective as a proliferation retardant than sulfoxide in cystine, because the group in this latter combination is more easily reducible by living tissue than is the former.

Two factors may be chiefly responsible for this difference in reducibility. The first is that methionine sulfoxide has but one inhibiting group while cystine disulfoxide has two. The second may well be the nature of the radicle to which the sulfoxide is attached. To present the argument for the first possibility would involve an excursion into chemical theory and speculation which is beyond the legitimate bounds of this paper. The reasonableness of the supposition will therefor be left to the judgment of the reader. Support for the idea that difference in the nature of the radicle may be a contributing factor is had from the data of Lavine (1936) for cystine

disulfoxide, and the data of Toennies and Kolb (1939) for methionine sulfoxide. These studies show that the two compounds differ markedly with respect to hydrolytic behavior *in vitro*. But it must be emphasized that these *in vitro* studies give no indication of the relative reducibility of the two compounds *in vivo;* for no chemical or biological reducing systems other than those provided by the compounds themselves were present, while in living tissue such are always present and determining a delicate and continuous balance between oxidation and reduction.

One more aspect of the results and I am done. Earlier reports have given the experimental evidence and argument for the possibility that a change from body cell to cancer cell precedes the appearance of a tumor and that this change is expression of cellular specialization or differentiation and not of proliferation (Hammett, 1936, 1940, 1941). If such be true it would be expected that sulfoxide would have no preventative influence, since this chemical group is primarily associated with proliferation. And such happened in this experiment, for the percentage of mice with multiple tumors was essentially the same in the *MSO* as in the control group. This can be taken, then, as evidence supporting the original thesis.

On the other hand there was a slight increase in the number of multiple tumors in the *MSO* mice. That is to say, *MSO* mice which could produce two tumors were able to produce more subsequent tumors than the controls. This is shown by the fact that the percentage of *MSO* mice with two tumors was less than control, while the percentage with three, four, and five tended to be greater. From the preceding paragraph it may be inferred that methionine sulfoxide was not a factor in increasing the number of anlagen. This is possibly determined by a constitutional disposition to multiple tumor formation. What methionine sulfoxide may have done was to provide an additional stimulus to proliferation expression of the anlagen already there by virtue of the reducing effect of increasing tumor mass as set forth in the general exposition. The fact that multiple tumor appearance was increased is at least not inconsistent with this thesis.

E. Summary and Conclusion

Sixty-five mice with spontaneous mammary tumors were daily injected with 0.094 millimols of dl-methionine sulfoxide from the day of appearance of their first tumors until death. Record was made of the age at which the tumors appeared, the number of tumors per mouse, the growth of the tumors in cc., the age of the mice at exitus, and

the changes in body weight. These were compared with like data from 76 control untreated mice of like sex, strain, environment, and diet.

A mixed reaction was produced by the sulfoxide which because of its consistency in relation to tumor genesis was valid expression of influence.

There was retardation of first tumor growth and prolongation of life in mice with one and two tumors. On the other hand there was acceleration of second and third tumor growth. The mixed reaction is attributable to the reducibility of sulfoxide attached to methionine in the presence of excess sulfhydryl derived from increasing masses of proliferating tumor tissue.

The conclusion is that whereas dl-methionine sulfoxide has no practical value as a retardant of tumor growth it is of great usefulness in the elucidation of the factors concerned in the intermediary metabolism of the sulfur groups.

F. Acknowledgment

Full credit should be given to Miss Jane Curtis and Miss Bernice Stevens for the computations, formulae, and charts.

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NOTES ON THE CO_2-REQUIREMENT OF BACTERIA*

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A. PROBLEM

The very remarkable observation that bacteria will not grow in the absence of carbon dioxide was proved beyond doubt by Valley and Rettger in 1927, and has been verified since by several other authors. This fact is so unusual because most bacteria produce CO_2 as a normal metabolic product and it seemed strange that its removal could prevent growth and multiplication. Besides, the need of CO_2 by the chlorophyll-free bacteria whose metabolism resembles that of animal cells in many respects seemed contradictory to our conceptions of cell metabolism. The recent investigations of Wood and Werkman (1938) have shown that considerable quantities of CO_2 can be assimilated at least by certain species, perhaps by all. This proves the possibility of CO_2 utilization, but not its necessity. It is the necessity that is considered in this paper.

The CO_2 production of most bacteria must be considerably larger than the CO_2 consumption, for if it were otherwise, we would not know about CO_2 production. If we want to prove CO_2 consumption, it will be necessary to remove the continually produced CO_2 as rapidly as it is formed. The success of the experiment depends evidently on the difference between rate of production and rate of removal. Since the metabolism of old cells is slower than that of rapidly multiplying cells, the experiments should be more successful with old cells. Apparently, Valley and Rettger as well as Walker (1932) and Gladstone, Fildes, and Richardson (1935) used always old cells immediately after being transferred for their experiments. It seemed worth investigating how young cells at the height of metabolism would react to CO_2 removal. The above experiments prove only that CO_2 is needed during the "lag phase," i.e., the period of rejuvenation of the old cells in a favorable environment.

Two other points were investigated. One concerns the effect of the amount of inoculum. With large numbers of cells, CO_2 production per cc. of culture is increased, and if the rate of removal remains unchanged, a large inoculum may grow where a small inocu-

*Received for publication on January 31, 1941.

lum fails. The other investigation concerns those species which are known not to produce CO_2. It should be possible to show that these organisms are more sensitive to CO_2 removal, and that neither large numbers nor young cells will make it possible for them to overcome lack of carbon dioxide.

B. Method

The use of agar plates by Valley and Rettger presented considerable experimental difficulties, and the aeration method by Walker (1932) and by Winslow, Walker, and Sutermeister (1932) was not adaptable to the comparative study of several different inocula of a number of species. The experiments reported here were made by cultivating the bacteria on coverglasses in droplets hanging over a drop of KOH. The CO_2 removal was very rapid, and a large number of parallel cultures could be made at once.

The concavity of a microculture slide was surrounded by a thin vaseline ring. At the bottom of the cavity, a tiny drop of KOH solution, 2.5 per cent, was placed. A small hanging drop of the culture to be tested was placed on a sterilized coverglass, and the slide pressed over this coverglass so that the hanging drop was completely shut off from the outside air by the vaseline. Controls were made with a 2.5 per cent solution of KCl to provide an approximately equal osmotic effect on the hanging drop.

All droplets were made with the same loop, and the average weight of 15 droplets was 2.8 mg. From this weight and from the plate counts of the bacterial population of the cultures, the number of bacteria per drop could be estimated. The size of the drops decreased, due to some distillation to the strong solution at the bottom of the cavity. The droplet weight after two days had decreased to 1.2-2 mg. With water in place of KCl, the drops increased to 6-9 mg. All experiments here reported are made with KCl as control.

The medium was in most cases glucose broth; the doubling of the concentration of the contents by the drying effect of KOH did not interfere with the growth of the bacteria tested. All cultures were kept at 30° C.

C. Results

1. *Young Cells versus Old Cells*

The metabolism of young cells is much more active than that of old cells. Huntington and Winslow (1937) found the CO_2 production of one-hour old bacteria 3 to 14 times as high as that of 24-hours

old cells. The peak of CO_2 production per cell is reached two to five hours after the transfer of old cells to a fresh medium.

In the experiments reported here, the old cells were 2 to 12 days old, while the young cells were mostly taken from cultures which had not yet become cloudy. Usually they came from 2- to 4-hours-old transfers from 12- to 14-hours-old cultures, and were at the peak of multiplication. Their metabolism was not interrupted by the experiment because all dilutions were made with the same glucose broth in which they were cultivated.

Table 1 shows that CO_2 removal could hardly prevent young cells

TABLE 1

PREVENTION OF THE GROWTH OF BACTERIA PRODUCING CO_2 BY THE REMOVAL OF CO_2
(+ means growth)

		Young cells			Old cells		
Bacterium coli	cells per drop	650	65	7	3,500	350	35
	growth over KCl	+	+	+	+	+	+
	growth over KOH	+	+	+	+	+	0
Achromobacter viscosum	cells per drop	?	?	?	26,000	2,600	260
	growth over KCl	+	+	+	+	+	+
	growth over KOH	+	+	+	+	+	0
Bacillus subtilis	cells per drop	8,400	840	84	160	16	2
	growth over KCl	+	+	+	+	+	+
	growth over KOH	+	+	+	+	0	+
Sarcina lutea	cells per drop	150	15	2	185	18	
	growth over KCl	+	—	+	+	0	
	growth over KOH	0	—	0	0	0	
Pseudomonas fluorescens	cells per drop	7,300	730	73	1,900	190	19
	growth over KCl	+	+	+	+	+	+
	growth over KOH	+	+	+	+	+	+
Saccharomyces cerevisiae	cells per drop	84	9	1	270	27	3
	growth over KCl	+	0	0	+	lost	+
	growth over KOH	+	0	0	+	+	0
Torula cremoris	cells per drop	1,200	120	12	220	22	2
	growth over KCl	+	+	+	+	+	+
	growth over KOH	+	+	0	0	+	0

from growing unless the cell number per drop was very small. Yet the examples of *Sarcina lutea*, and the yeast show that with less than 10 cells per drop, growth can be prevented by KOH. This is important because it demonstrates that bacteria need CO_2 not only during the lag phase, for rejuvenation, but during their entire multiplication period.

A very frequent observation was a considerable retardation of growth by KOH in those cultures which gave ultimately positive

growth. The result of the controls was final after 24 hours, while the droplets over alkali often showed no growth at this time, or only so few cells that they might be considered to be the original inoculum; but on the following day, the droplet was crowded.

2. *Large versus Small Inocula*

The prediction that large cell concentrations might produce CO_2 more rapidly than it could be removed, and might therefore develop in presence of KOH while small inocula fail, is borne out in Table 2.

TABLE 2

SIZE OF INOCULUM AND GROWTH WITHOUT CO_2

	Number of cells inoculated per droplet					
	Over 100,000	10,000 to 99,999	1,000 to 9,999	100 to 999	10 to 99	1 to 9	Less than 1
				Old cells			
Bacterium coli		0	0	0	0		
Bacterium coli			+	+	0		
Bacterium herbicola			+	+	+		
Achromobacter viscosum		+	+	0			
Bacillus subtilis				+	0	+	
Bacillus cereus				+	+	+	
Bacillus cereus				0	0	0	0
Sarcina lutea				0		0	
Sarcina lutea				0	0	0	0
Pseudomonas fluorescens	+	+	+	+	+		
Saccharomyces cerevisiae				+	+	0	
Torula cremoris				0	+	0	
Total growth	1	2	4	6	5	2	0
Total no growth	0	1	1	6	5	5	2
				Young cells			
Bacterium coli				+	+	+	
Bacillus subtilis			+	+	+		
Sarcina lutea				0	0	0	
Pseudomonas fluorescens			+	+	+		
Saccharomyces cerevisiae					+	0	0
Torula cremoris				+	+	0	
Total growth			2	4	5	1	0
Total no growth			0	1	1	3	1

While different organisms disagree in their limits, the summary shows very plainly that the probability of growth decreases as the number of cells per drop decreases. The ratio of growing to not growing cultures is different between young and old cells as would be expected. This ratio can be expressed as "percentage of tests showing growth." This permits the following comparison:

Order of magnitude of inoculum:	10^6	10^4	10^3	10^2	10^1	10^0	10^{-1}
Percentage growth, old cells:	100	67	80	50	50	28	0
Percentage growth, young cells:	—	—	100	80	83	25	0

The advantage of a large inoculum in rapidly producing the necessary CO_2 would seem to give a good explanation for the observation that the duration of the lag period is shorter with larger inocula. Walker (1932) and Winslow and Walker (1939, p. 157) consider this an important factor. However, Rahn (1933) and Herrington (1934) have shown that the lag time is proportional to the logarithm of the inoculum while the CO_2 production must necessarily be proportional to the number of cells. There must be another, more important factor controlling the lag time of bacteria.

3. *Bacteria Which Do Not Produce* CO_2

Some species do not produce CO_2, and if they require CO_2 like all other species, they must suffer more from CO_2 removal than those which can at least partly make up for this loss. With them, large inocula would have no advantage over small inocula, and young cells would have no advantage over old ones. Tables 3 shows some experiments with two species of *Lactobacillus* and one of *Streptococcus* which are in agreement with this conclusion. Only twice was growth observed, and one of these two observations may be due to contamination. Both occurred with inocula of more than 10,000 cells. Young cells show no advantage over old cells.

TABLE 3

PREVENTION OF GROWTH BY REMOVAL OF CO_2 FROM BACTERIA UNABLE TO PRODUCE CO_2

		Young cells			Old Cells		
Lactobacillus acidophilus	cells per drop	200	20	2	300	30	3
	growth over KCl	+	+	0	+	+	+
	growth over KOH	0	0	0	0	0	0
Lactobacillus casei	cells per drop	75	8	1	50,000	5,000	500
	growth over KCl	+	+	0	+	+	+
	growth over KOH	0	0	0	+*	0	0
Streptococcus lactis	cells per drop	12,000	1,200	120	?	?	?
	growth over KCl	+	+	+	+	0	0
	growth over KOH	+**	0	0	0	0	0
Streptococcus lactis	cells per drop				900	90	9
	growth over KCl	—	—	—	+	+	0
	growth over KOH				0	0	0

*Growth very poor compared with KCl control.
**Cells larger than in control, many abnormal form; perhaps contaminated.

The greater CO_2 need of this group of organisms is quite evident

from Valley and Rettger's experiments on the growth with excess CO_2, 0.3 to 1.0 per cent. Duplicate agar plates were kept in air, and in air + CO_2. The latter developed far more colonies, and the average increase through CO_2 was 56 per cent with those bacteria which produced CO_2. But with *Lactobacillus acidophilus*, the average of four experiments gave an increase of 180 per cent.

D. Summary

Bacteria need carbon dioxide not only during the lag phase for purposes of rejuvenation, but also during the period of active growth. Their metabolism at this stage of development is so great that the need of CO_2 can be proved only when very few cells are inoculated.

With those bacterial species which produce CO_2 as a metabolic product, the need of CO_2 does not become evident if large numbers of cells are tested. In this case, growth and multiplication are greatly retarded, but not completely prevented.

Species which do not produce CO_2 in their metabolism react more promptly to the removal of CO_2. Even large inocula and young cells cannot overcome the absence of CO_2.

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